Chemical changes and off-flavor development in lactose-hydrolyzed UHT milk during storage

PhD Thesis By

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Abstract

The awareness of lactose intolerance has increased tremendously the last years and a comparable increased demand for lactose-hydrolyzed dairy products has been observed. Lactose-hydrolyzed milk is produced by reducing the lactose content by filtration to approximately 60% of original level of lactose, followed by an enzymatic hydrolysation of the remaining lactose in the milk by the enzyme preparation, consisting mainly of β-galactosidase. To increase the shelf-life of the milk, the milk is heat treated, and one heat treatment is ultra-high temperature (UHT) heating. During heating several reactions in the milk are initiated, one of them is the Maillard reaction. This reaction has a major effect on the appearance and quality of the milk and must be avoided in milk to keep the high quality during storage.

The overall aim of the present study was to elucidate the chemical changes that occur in lactose-hydrolyzed UHT milk during nine months of storage. These chemical changes were expected to be the reason for the formation of off-flavor observed in lactose-hydrolyzed milk after approximately 90 days of storage. The specific aims of the study were 1) to compare lactose-hydrolyzed and conventional UHT milk during storage, 2) to identify specific compounds correlated to the Maillard reaction, 3) to investigate the changes in protein profile in lactose-hydrolyzed and conventional UHT milk during storage, and finally 4) to evaluate how the chemical changes affected the sensory profile of the milk.

Paper 1 describes the method applied to analyze volatile compounds in the milk. Dynamic headspace sampling in combination with gas chromatography mass spectrometry (DHS-GC-MS) was used due to its holistic way of analyzing volatiles. The study showed that DHS-GC-MS was a useful analytical method, and in total 24 volatile compounds were identified in the milk. The results revealed a variation between batches of the same milk type, especially in the level of ketones. Moreover, the ketone concentration was observed to be higher in the conventional UHT milk compared to the lactose-hydrolyzed UHT milk.

Paper 2 included several methods to investigate the chemical changes in milk during storage. Among others, DHS-GC-MS, reversed phase high pressure liquid chromatography mass spectrometry (RP HPLC MS), and a fluorescamine assay were used to investigate volatile compounds, furosine, and free amino terminals respectively. The major results were that a lower initial level of 2-methylbutanal, furosine and the free amino terminals were observed in lactose-hydrolyzed milk compared to the conventional milk. However, during storage the level of these
compounds increased significantly in the lactose-hydrolyzed milk, which was due to increased reactivity of the lactose hydrolysation products glucose and galactose.

Paper 3 elucidates the protein hydrolysation in the milk by HPLC MS and 1H NMR spectroscopy. Elevated levels of free amino acids were identified in the lactose-hydrolyzed milk during storage while the levels remained constant in the conventional milk. Proteolysis were observed in all milk types during storage, however, the protein hydrolysis was more evident in the lactose-hydrolyzed milk compared to the conventional milk, especially in β-CN and αs1-CN after 90 days of storage.

Paper 4 evaluated the flavor, taste and aroma of the milk with sensory descriptive analysis, which was correlated to the volatile compounds analyzed with DHS-GC-MS. The stale flavor increased in all milk types and could be correlated with increased level of ketones and the aldehydes 2-methylbutanal, octanal and decanal. A higher level of bitter taste was observed in the lactose-hydrolyzed milk than in the conventional milk, and a correlation with the level of free amino terminals analyzed with the fluorescamine assay revealed a good link between bitter taste and the level of free amino terminals in the milk.

In conclusion, this present study showed that lactose-hydrolyzed UHT milk is more vulnerable towards chemical changes compared to conventional UHT milk. This is due to increased reactivity of glucose and galactose, and due to the proteolytic activity present in the enzyme preparation added to the lactose-hydrolyzed milk. The proteolytic enzymes hydrolyses the proteins to bitter tasting peptides and to amino acids that are included in the Maillard reaction to form flavor compounds such as Strecker aldehydes.
Resume


Det overordnede formål med dette projekt var at undersøge de kemiske forandringer der forekommer i laktose-hydrolyseret UHT mælk under ni måneders opbevaring. Disse kemiske forandringer forventedes at være årsagen til dannelsen af off-flavorer opdaget i laktose-hydrolyseret mælk efter omkring 90 dage opbevaring. De specifikke delmål med projektet var 1) at sammenligne laktose-hydrolyseret og konventionell UHT mælk under opbevaring, 2) at identificere specifikke forbindelser der var korrelerede til Maillard reaktionen, 3) at undersøge ændringerne i protein profilen i laktose-hydrolyseret og almindelig UHT mælk under opbevaring, og til sidst 4) at evaluere hvordan de kemiske ændringer påvirkede den sensoriske profil af mælken.

Artikel 1 beskriver den analytiske metode anvendt til at analysere de flygtige forbindelser i mælken. Dynamisk headspade sampling i kombination med gas kromatografi mass spektrometri (DHS-GC-MS) anvendtes for dens holistiske måde at analysere på. DHS-GC-MS viste sig at være en anvendelig analytisk metode, og der identificeredes i alt 24 flygtige forbindelser i mælken. Resultaterne viste, at en variation mellem batch af den samme mælketype forekom, specielt i niveauet af ketoner. Derudover, observeredes en højere koncentration af ketoner i den almindelige UHT mælk sammenlignet med den laktose-hydrolyserede mælk.

Artikel 2 inkluderede adskillige metoder til at undersøge de kemiske ændringer i mælken under opbevaring. Bland andet anvendtes DHS-GC-MS, reversed phase high pressure liquid chromatography mass spectrometry (RP HPLC MS), og et fluorescamine assay for at undersøge de flygtige forbindelser, furosine og de frie amino terminaler. De primære resultater var, at et lavere start niveauet af 2-methylbutanal, furosine og de frie amino terminaler var observeret i den laktose-
hydrolyserede UHT mælk sammenlignet med den almindelige UHT mælk, dog øgedes koncentrationen af disse forbindelser signifikant i den laktose-hydrolyserede mælk under opbevaring, grundet en højere reaktivitet af laktose hydrolyserings produkterne glukose og galaktose.

Artikel 3 belyste protein hydrolysen i mælken ved at anvende HPLC MS og kernemagnetisk resonans (NMR) spektroskopi. Øgede mængder af frie amino syrer var konstateret i den laktose-hydrolyserede mælk under opbevaring mens mængde forblev konstant i den almindelige UHT mælk. Proteolyse var påvist i alle mælke typerne under opbevaring, men protein hydrolyseringen var mere tydelig i den laktose-hydrolyserede mælk i forhold til den almindelige mælk, specielt i β-CN og α₁s-CN efter 90 dages opbevaring.

Artikel 4 evaluerede flavor og smag i mælken ved brug af sensorisk deskriptiv analyse, som korreleredes med de flygtige forbindelser i mælken, analyseret med DHS-GC-MS. Den hengemte flavor øgedes i alle mælke typer og korreleredes med et forøget niveau af ketoner og følgende aldehyder: 2-methylbutanal, octanal og decanal. Et forhøjet niveau af bitter smag var bemærket i den laktose-hydrolyserede mælk i forhold til den almindelige mælk, som korreleredes med niveauet af frie amino terminaler analyseret med fluroscamine assayet. Der blev fundet en god korrelation mellem bitter smag og niveauet af frie amino terminaler i mælken.

Konklusionen på dette projekt er, at laktose-hydrolyseret UHT mælk er mere sårbar i forhold til kemiske forandringer sammenlignet med almindelig UHT mælk. Dette skyldes en øget reaktivitet af glukose og galaktose og en øget proteolytisk aktivitet til stede i enzym præparationen som tilføjedes til den laktose-hydrolyserede mælken. Det proteolytiske enzyme hydrolyserer proteinerne i mælken til bitre peptider og til amino syrer som kan inkluderes i Maillard reaktionen og danne flavor forbindelser såsom Strecker aldhyder.
List of publications

Paper 1  Volatile Component Profiles of Conventional and Lactose-Hydrolyzed UHT Milk – A Dynamic Headspace Gas Chromatography-Mass Spectrometry Study
*Dairy Science and Technology (2014) 94:311-325*

Paper 2  Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional UHT milk
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Paper 3  Chemical and proteolysis-derived changes during long-term storage of lactose-hydrolyzed UHT milk
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Paper 4  Storage-induced changes in the sensory properties of conventional and lactose-hydrolyzed milk explained by the development of volatiles
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Abbreviations

AGEs; Advanced glycated end products; α-CN, alpha-casein; β-CN, beta-casein; κ-CN, kappa-casein; CONVI, Conventional indirect UHT; DHS, Dynamic headspace sampling; DMDS, Dimethyl disulfide; DMTS, Dimethyl trisulfide; ESL, Extended shelf life; GC-MS, Gas chromatography-mass spectrometry; LA, Lobry de Bruyn-van Ekenstein; α-LA, Alfa-lactalbumin; β-LG, Beta-lactoglobulin; LHD, Lactose-hydrolyzed direct UHT; LHI, Lactose-hydrolyzed direct UHT; LPH, Lactase-phlorizin hydrolase; MS, mass spectrometry; NF, Nanofiltration; NMR, Nuclear magnetic resonance; OH, hydroxyl; PLS, Partial least square; RP HPLC, Reversed phase high pressure-liquid chromatography; SDS-page, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SIM, selected-ion monitoring; SPME, Solid phase micro extraction TAG; Triacylglycerol; Tg, Glass transition temperature; TIC, Total ion current; TSP, Sodium trimethylsilyl-[2,2,3,3-2H4]-1-propionate; UF, Ultrafiltration; UHT, Ultra high temperature; US, United States; UV, Ultraviolet
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Introduction

Milk contains 87% water but is one of the most nutrient-dense foods, containing the caseins (CN) and whey proteins, lipids, the disaccharide lactose, minerals and vitamins (Walstra et al. 1999). In addition, milk contains bioactive peptides (Choi et al. 2012), immunoglobulins and oligosaccharides which make the milk a good nutrient source for infants as well as for adults.

Approximately 70% of the world population is lactose-intolerant. Lactose-intolerant people lack the production of lactase by the highly specific epithelial cells found in the small intestine. The lack of the lactase enzyme leads to an inability to hydrolyze lactose to glucose and galactose, which in turn is the cause of unpleasant problems, such as abdominal pain and diarrhea (Heyman 2006). Lactase persistence is genetically determined and the ability to produce lactase has developed from positive natural selection in countries where milk became domesticated over 7000 years ago (Ingram et al. 2009; Sverrisdóttir et al. 2014). Due to the high prevalence of the world population suffering from lactose-intolerance, lactose-hydrolyzed milk is now commercially produced, containing 0.01% lactose compared to the original level 4.8%. The production of lactose-hydrolyzed milk includes filtration that reduces the level of lactose in the milk to approximately 60% of original level, followed by addition of a β-galactosidase enzyme (lactase) preparation that hydrolyzes the remaining lactose to glucose and galactose. The enzyme can originate from bacteria, yeast or fungi and the most used enzyme is isolated from the yeast Kluyveromyces or the fungi Aspergillus (Ansari and Satar 2012). The enzymes used for hydrolyzing lactose in milk are differentiated by their purity, and a highly pure enzyme is expected to contain only β-galactosidase. Mittal et al. (1991) investigated the importance of the enzyme preparation purity and observed a higher proteolytic activity in the less pure preparation (Mittal et al. 1991).

In order to increase the shelf-life of milk, different heat treatments are applied such as pasteurization (72 °C, 15 sec), extended shelf-life (ESL) (130-145, <1 sec) and ultra-high-temperature (UHT) (135-150 °C, 2–20 sec.) treatments (Walstra et al. 1999; Kessler 2002; Rysstad and Kolstad 2006). In UHT milk the bacteria are killed and most of the indigenous enzymes inactivated, which gives the milk a long shelf-life at ambient storage temperature. The market for UHT treated milk is mainly in Europe as well as Asia. Asia does not have a tradition for daily intake of milk, however, the positive effects associated with milk intake have increased the market considerably in Asian countries and the demand for milk products has increased tremendously in the past years (Beghin 2006; Fuller et
Introduction

al. 2006). However, most of the population in Asia is lactose-intolerant, which increases the demand for high-quality lactose-hydrolyzed milk products.

The enzyme preparations used in the lactose-hydrolyzed milk has shown to contain side-activities that hydrolyze the proteins in the milk during storage (Mittal et al. 1991; De et al. 2007). The hydrolysis of proteins may have major effects on texture, taste and flavor due to formation of peptides, which can be bitter-tasting (McKellar et al. 1984; Harwalkar et al. 1993; Gomez et al. 1997), and free amino acids that can be generated by the hydrolysis process. The lysine residues and amino terminals of intact proteins and peptides, as well as free amino acids, can participate in the Maillard reaction as well as other reactions. The Maillard reaction is a non-enzymatic, heat-initiated reaction between ε-amino groups of proteins, peptides or free amino acids and the carbonyl group of reduced carbohydrates present in the milk. The Maillard reaction has major effects on the appearance and quality of milk and may decrease the shelf-life of the milk. Therefore, the Maillard reaction is not desired in milk and should be avoided if possible. It is well established that the lactose hydrolysates, i.e. glucose and galactose reacts with proteins to a higher degree than lactose itself (Kato et al. 1986; Kato et al. 1988; Meltretter et al. 2009; Naranjo et al. 2013), which consequently increases the prevalence for formation of Maillard reaction products in lactose-hydrolyzed UHT milk as compared with conventional UHT milk during storage.

The shelf-life of ambient temperature stored lactose-hydrolyzed UHT milk is three months, which is remarkably lower than the eight month shelf-life of conventional UHT milk. The shelf-life is limited, due to an observed formation of off-flavor, which is not well defined or described, but decreases the consumer acceptance of the milk. The sensory attributes, and primarily flavor, are crucial for the consumer acceptance of milk and should not be compromised (Thomas 1981; Oliver 1993; Adhikari et al. 2010). Therefore, it is essential to investigate this off-flavor formed in lactose-hydrolyzed UHT milk, to increase the understanding of its origin and how to decrease its occurrence. In the present study chemical changes in the volatile, protein, metabolic and proteomic profiles were investigated in lactose-hydrolyzed, filtered and conventional UHT milk to determine the chemical changes that takes place. In addition, a sensory descriptive analysis was applied to elucidate how the chemical changes affected the sensory characteristics of the milk during storage.

Since the beginning of this project in 2011 the production and sale of lactose-hydrolyzed milk has increased tremendously in Denmark, and the market is expected to continue to grow nationally and internationally. In Denmark alone,
the sale of lactose-hydrolyzed milk increased with 500% in 2011 to 2012, and the sale of lactose-hydrolyzed products continued to increase in 2013 with 113%, and a 70% increase is expected in 2014 to 2015 (Thalbitzer 2012; Tüchsen 2014).

**Aims and hypotheses**

The overall aim of this PhD project was to elucidate the chemistry behind the formation of off-flavor in lactose-hydrolyzed UHT milk and to identify differences in the volatile, metabolic, proteomic and sensory profile between lactose-hydrolyzed and conventional UHT milk during storage.

The specific aims of the study were

1. to compare lactose-hydrolyzed and conventional UHT milk during storage
2. to identify specific compounds linked to the Maillard reaction crucial for the changes in chemical composition in lactose-hydrolyzed UHT milk using DHS-GC-MS and RP HPLC MS
3. to investigate the changes in the protein profile in lactose-hydrolyzed and conventional UHT milk during storage, using RP-HPLC MS and fluorescamine assay
4. to evaluate how chemical changes in the lactose-hydrolyzed UHT milk affect the sensory characteristics compared to conventional UHT milk using sensory descriptive analysis

It is hypothesized that lactose-hydrolyzed UHT milk is more prone to Maillard reactions compared to conventional milk due to a higher reactivity of glucose and galactose with proteins. In addition, it is hypothesized that the enzyme β-galactosidase used in the present study contains side activities from proteolytic enzymes hydrolyzing the proteins in the lactose-hydrolyzed milk that contribute to off-flavor formation. Finally, it is hypothesized that a higher level of Maillard reaction products and formed peptides change the sensory characteristics in lactose-hydrolyzed milk compared to conventional UHT milk.

**Outline of the thesis**

The overall purpose of this thesis is to present the results obtained in this PhD study in relation to the existing research within the field. In chapter 1 a brief overview of the major milk components (carbohydrates, protein and lipids) are
discussed, in chapter 2 common heat treatments applied to milk products is considered and in chapter 3 the occurrence of lactose intolerance and the properties of the enzyme β-galactosidase is discussed. In chapter 4 the methods used to analyze the milk in the present study is briefly discussed. However, the dynamic headspace sampling gas chromatography-mass spectrometry method was optimized prior to the analyzing of volatile compounds in the milk and will therefore be thoroughly discussed. The co-authors on the papers have contributed to the RP-HPLC MS analysis of the proteins, the HPLC analysis of furosine and the 1H NMR spectroscopy analysis of the metabolites (paper 2, 3 and 4). In chapter 5 the Maillard reaction and glycosylation are described and the results obtained from the present study related to the Maillard reaction product formation are presented (paper 2 and 3). In chapter 6, proteolysis is reviewed and differences in proteolytic activity between the lactose-hydrolyzed and conventional UHT milk are addressed (paper 2 and 3). Chapter 7 gives an overview of the lipid oxidation reactions present in the milk (paper 1, 2 and 3). Chapter 8 presents the relevance of aroma and flavour changes in milk and the results from the sensory descriptive analysis of lactose-hydrolyzed and conventional UHT milk (paper 4). Furthermore, in chapter 9 the effect of processing of lactose-hydrolyzed UHT milk is discussed in a holistic perspective including the most important results from paper 1, 2, 3 and 4. Finally, in chapter 10, conclusions and future perspectives of the results obtained in the PhD study are presented.

An overview of the workflow in this PhD project is presented in Figure 1.

Figure 1. Overview of the milk and analyzing methods included in the present study.
Chapter 1. Milk

In this chapter the carbohydrates, proteins and lipids present in the milk will be reviewed.

1.1 Carbohydrates

The carbohydrate in milk is mainly lactose which is a disaccharide consisting of the monosaccharides glucose and galactose bound together by a β-1,4-glycosidic bond. The main purpose of lactose in milk is to supply the infant with energy, in addition, lactose retains the osmotic pressure between the milk and blood. The concentration of lactose in bovine milk is approximately 4.8% (Fox 2009), but varies throughout the lactation with an observed higher content of lactose in the early stage of lactation (Walstra et al. 1999). Moreover, milk also contain trace amounts of glucose and galactose (Sundekilde et al. 2013a).

Lactose, glucose and galactose are reducing carbohydrates, and therefore contain a free carbonyl group such as an aldehyde (Figure 2). The D-form of the monosaccharides can have three different steric structures, an open-chain form and two different cyclic forms (pyranose). When the carbohydrate molecule is dissolved in water the cyclic forms are converted to each other via the open-chain form, by the reaction between the aldehyde (C1) and the alcohol (C5) in the molecule. The cyclization leads to an asymmetric centre and therefore the cyclic form consists of two isomers, the α- and β-anomer. The conformations are present at different levels depending on the thermodynamically most preferable and stable form. The most preferable and stable forms for glucose and galactose are the cyclic chair conformation of the β-anomer, and the least preferable is the acyclic, open-chain aldehyde, form (Yaylayan et al. 1993; Belitz et al. 2001). The chair conformation is most preferable due to the equatorial positions of hydroxyl (OH) and CH2OH groups, which decreases the interaction between the groups as compared to the axial positions, and thereby increases the stability of the molecule. In addition, the configuration of carbon three and four in the glucose and galactose molecule have shown to be important for their reactivity. It has been revealed that galactose has an equatorial chair configuration at carbon three and four, while glucose has the less energetically preferable axial chair configuration (Kato et al. 1986; Kato et al. 1988) (Figure 3). Consequently, when galactose is condensed with a protein, this galactose-protein product is more prone to degrade into polymers compared with similar glucose-protein products. In the lactose molecule, the galactose molecule is attached to the
glucose at the hydroxyl group at carbon four which limits further degradations towards brown compounds and polymers.

The rotation between the different conformations occurs at a specific reaction rate $K$ which is highly temperature and somewhat pH dependent, and the concentration of the open-chain aldehyde in the solution increases with increasing temperature and is preferred at alkaline pH (Walstra et al. 1999). The reactivity of the carbohydrate molecule is highly dependent on the acyclic form, thus, the higher level of the acyclic form the higher reactivity of the carbohydrate (Bunn and Higgins 1981; Yaylayan et al. 1993; Brands et al. 2002; Naranjo et al. 2013). The level of acyclic form of the carbohydrates present in milk is as follows: galactose>glucose>lactose (Hayward 1977). Furthermore, Naranjo et al (2013) discussed that the reaction rate constant for the condensation of a specific carbohydrate and a protein were affected by the molecular mobility of the carbohydrate which was dependent on the glass transition temperature ($T_g$) (Naranjo et al. 2013). Thus, the reaction rate of the carbohydrate increases with increasing difference between the storage temperature and $T_g$. Consequently, lactose with higher $T_g$ than glucose and galactose will react with a protein with a lower reaction rate compared with glucose and galactose at room temperature.

**Figure 2 Mutarotation of glucose.**

**Figure 3 Configuration of sugar-protein Amadori product.** $R=$Galactose (Kato et al. 1986; Kato et al. 1988)
Carbohydrates give a sweet taste to milk, and different carbohydrates can be perceived differently. At a scale of perceived sweetness where saccharose is the reference substance (relative sweetness 100), fructose has a high sweetness (relative sweetness 114), followed by glucose (relative sweetness 69), galactose (relative sweetness 63), and the disaccharide lactose (relative sweetness 39) is one of the least sweet carbohydrates (Belitz et al. 2001).

The carbohydrates are included in different reactions during extensive heating, in acid and alkaline conditions. Glucose, mannose and fructose are in equilibrium via the 1,2-enediol, and fructose is in equilibrium with 2,3-enediol, this enolization and the subsequent isomerization is termed as the Lobry de Bruyn-van Ekenstein rearrangement (LA-transformation). Lactose is also included in the LA-transformation and is in equilibrium with the 1,2-enediol and lactulose. The formed enediols may react further via β-elimination to the highly reactive dicarbonyl compounds.

Carbohydrates are also included in the Maillard reaction, which is discussed in chapter 5.

1.2 Proteins

The two major protein groups in milk are caseins (CN) and whey proteins, and milk protein comprises approximately 3.5% (w/w) of bovine milk composition (Walstra et al. 1999). In total, 80% (w/w) of the proteins are caseins, which are divided into four main groups; αS1-casein (αS1-CN), β-casein (β-CN), αS2-casein (αS2-CN) and κ-casein (κ-CN), where αS1-CN and β-CN are the most abundant (Wang et al. 2009). The remaining 20% of the proteins is whey proteins, primarily β-lactoglobulin (β-LG) (50%) and α-lactalbumin (α-LA) (20%), but also immunoglobulins and enzymes (Farrell et al. 2004). CN has a low degree of secondary and tertiary structure, and most of the casein molecules are found as aggregates forming micelles, which are kept together by hydrogen bonds, hydrophobic interactions and electrostatic interactions (Dalgleish and Corredig 2012). β-LG is a globular protein that depending on pH and temperature can be present as dimer or monomer. The primary structure of β-LG is composed of 162 amino acids comprising five cysteines forming two disulfide bonds and a free thiol group (Cys121) which are hidden in a hydrophobic cleft of the protein and thereby not available for interactions with other proteins in the native form (Kontopidis et al. 2004).
Correlations between peptides and bitter taste have been observed in UHT milk (McKellar 1981; McKellar et al. 1984) (paper 4), and the nature of the amino acids included in the peptides has a major role for the perceived taste. The protein itself is not bitter, however, especially β- and αS1-CN contain a high level of hydrophobic amino acids, which, depending on conformation and length of the polypeptide they are located in, contribute to a bitter taste (Guigoz and Solms 1976; Lemieux and Simard 1992; Gomez et al. 1997; Kilara and Panyam 2003). Properties correlated to bitterness, in addition to the hydrophobicity, is aromatic side-chains, the presence of ammonium groups and the configuration of the α-carbon atom and the amino acids in the peptide. Among the bitter amino acids are isoleucine, leucine, lysine, methionine, phenylalanine, proline, tyrosine, tryptophan and valine (Lemieux and Simard 1992). The level of peptides in milk may increase with severe heat treatment and during long-term storage (Meltretter et al. 2008), which is due to both denaturation of the milk proteins as well as heat inactivation of inhibitors of the plasmin system in milk and thereby promoting hydrolysis of the proteins after heat treatment (Rauh et al. 2014).

The proteins are, as the carbohydrates, included in the Maillard reaction (discussed in chapter 5). In addition, the proteins are exposed for proteolysis which is discussed in chapter 6.
1.3 Lipids

Lipids present in milk are mainly triglycerides which are composed of a glycerol core based on three carbons. One fatty acid is esterified to each carbon atom (Figure 4) which can be either a saturated fatty acids, mainly unbranched and straight hydrocarbon chains, or unsaturated fatty acids, hydrocarbon chain containing up to four double bonds. In addition, the fatty acids can be replaced with β-keto acids (Hawke 1966). The triglycerides are apolar and are mainly found in the core of fat globules which are surrounded by a membrane containing polar phospholipids and proteins such as glycoproteins and enzymes (Walstra et al. 1999). Approximately 69% of the fat in milk is saturated, 27% monosaturated, and 3.3% polyunsaturated fatty acids. Especially, the unsaturated fatty acids and β-keto acids are of interest due to their high reactivity during heating and storage (Hawke 1966). The double bond in the unsaturated fatty acids is vulnerable towards oxidation, furthermore the hydrogen in the methylene group which is adjacent to two carbonyl groups in the β-keto acid is highly reactive (Whitfield and Mottram 1992).

Figure 4. Triglyceride consisting of a glycerol core attached to, from the top, palmitic acid, oleic acid and linolenic acid.

Lipids can be exposed to autoxidation and thermal degradation, which is discussed in chapter 7.
Chapter 2. Heat treatment

In this chapter the heat treatment of milk will be discussed including a brief discussion of the results obtained in the present study in relation to different heat treatments.

2.1 Heat treatment

Heat is applied to milk to kill bacteria and inactivate enzymes and thereby increase the shelf-life of the milk. The shelf-life is highly dependent on the severity of the heat applied to the milk, where the combination of temperature and time is essential. Two gentle heat treatments include pasteurization and extended shelf life (ESL) treatment. Pasteurization, 70-75 °C for 15-30 seconds, does not inactivate all the enzymes and therefore the shelf-life is approximately one week for this type of milk when stored cold. ESL treatment is a more severe heat treatment (130-145 °C for less than one second) than pasteurization. The ESL heated milk has almost the same appearance and taste as the pasteurized milk, but with a longer shelf-life of up to one month when stored at cold temperatures. Ultra high temperature (UHT) treatment (135-150 °C for 2-20 seconds) (Kessler 2002) is applied to inactivate a high level of enzymes present in the milk, and UHT-treated milk can be stored at ambient temperatures for several months. A disadvantage related to heating the milk at high temperatures is that the nutritional and sensory quality decreases due to denaturation of proteins, thermal degradations of lipids and reactions between sugar and proteins (Maillard reaction). Therefore, the combination of temperature and time of the heat treatment must be as low and short as possible to keep the high nutritional value and sensory quality of the milk, but still be able to inactivate the bacteria and enzymes present in the milk (Datta et al. 2002; Elliott et al. 2003).

2.2 UHT Process

An overview of a direct and indirect UHT process of milk is seen in Figure 5.
The UHT treatment can be divided into direct and indirect heat treatment. In direct UHT treatment steam is injected or infused into the milk, and in the indirect UHT treatment high-performance heat exchangers (plate or tubular) are used and the milk is not in contact with the heating media (steam or water). The tubular heat exchanger is preferred for UHT milk compared to plate heat exchangers due to the capability of higher pressure and temperatures (Walstra et al. 1999; Tetra Pak 2003). A slightly higher heating temperature (3-4 °C) is reached for the direct heat treatment to obtain the same bactericidal effect as the indirect heat treatment, but the direct treatment is gentler to the milk due to the shorter heating and cooling profiles compared to the indirect treatment (Figure 6). The shorter cooling times can be obtained due to the usage of a vacuum chamber, where additional water from the applied steam during heating, is also vaporized and removed (Datta et al. 2002).
2.3 Direct and indirect heat treatment of milk

Differences in milk composition have been observed between the two types of UHT milk. Lower levels of lactulose, furosine, β-LG denaturation and ketones have been observed in direct treated UHT milk compared to indirect UHT milk (Elliott et al. 2003; Perkins and Elliott 2005). In addition, a flavor less cooked has been reported in direct UHT milk, compared to the cooked flavor of indirect UHT milk (Datta et al. 2002). However, even if less chemical and sensory attribute changes have been observed in direct treated UHT milk the indirect treatment is preferred due to lower processing costs. In direct UHT treatment the steam used for heating cannot be reused and the energy applied is lost, while for the indirect UHT treatment the heating media can be reused, which decrease both the energy for heating and the consumption of water (Peterson et al. 2007).

Few studies have investigated the effect of different heat treatments on lactose-hydrolyzed UHT milk (Mendoza et al. 2005; Messia et al. 2007). Mendoza et al. (2005) investigated the level of furosine, lactulose and the carbohydrates glucose, galactose, fructose and tagatose as a function of heat treatment and time in lactose-hydrolyzed UHT milk. The milk was heated at 135, 140 and 150 °C for up to 40 seconds, and an increased level of furosine, fructose and tagatose was observed in the lactose-hydrolyzed milk with increasing heating temperature and time. Furthermore, the level of glucose and galactose decreased with increased temperature and time. In addition, Messia et al. (2007) investigated the level of furosine, lactulose and fructose in 14 different commercial lactose-hydrolyzed milk samples exposed to pasteurization and UHT heating. The level of furosine, lactulose and fructose was higher in the UHT treated lactose-hydrolyzed milk compared to the pasteurized lactose-hydrolyzed milk. In addition, the milk exposed to direct UHT treatment applied after hydrolysis of lactose resulted in a lower level of furosine and lactulose compared to the milk exposed to indirect UHT treatment applied before lactose hydrolysis.

In the present study milk exposed to direct and indirect UHT treatment applied after lactose hydrolysis was investigated (paper 2 and 3). No differences in volatile compounds, furosine or proteolytic activity were observed between the two heat treatments. However, a minor effect of heat treatment was presented in paper 4:table 2 where a significantly higher level of glycoprotein was observed in the indirect heated lactose-hydrolyzed UHT milk compared with the direct heated lactose-hydrolyzed UHT milk. From these results it was concluded that the reactions taking place in the lactose-hydrolyzed milk due to increased reactivity of glucose and galactose, compared to lactose, and increased proteolytic activity is more important for the chemical changes in lactose-
hydrolyzed UHT milk than the severity of heat treatment. The results considering the volatile compounds and the furosine formation during storage is further discussed in chapter 5, and the proteolysis results are presented in chapter 6.

2.4 The effect of heat treatment on proteins

The effect of heat treatment on milk proteins induces is structural changes including unfolding, rearrangement of disulfide bonds, denaturation and aggregations as well as glycation (de Wit 2009). The non-globular structure of CN makes the protein heat stable and CN is therefore only slightly affected by heat. β-LG, on the other hand, is highly dependent on the environmental conditions, and aggregates with different morphology and structure are formed during heating and pH changes (Kontopidis et al. 2004; Da Silva Pinto et al. 2012). When β-LG is heated the monomer form is favored (Zúñiga et al. 2010), which unfolds and exposes the free thiol group (121Cys) which subsequently reacts with other disulfide bonds in the same β-LG, another β-LG molecule, α-LA, κ-CN, αs2-CN, or MFGM proteins leading to the formation of small dimers and bigger clusters. During storage the disulfide linked aggregates undergo further aggregations via non-covalent bonding (hydrophobic interactions), which is favored when the pH and approximate the isoelectric point.
Chapter 3. Lactose-hydrolyzed milk

In this chapter lactose-intolerance, the production of lactose-hydrolyzed milk and the enzyme β-galactosidase will be discussed.

3.1 Lactose Intolerance

In humans lactose is hydrolyzed by the enzyme lactase-phlorizin hydrolase (LPH) produced by the epithelial cells present in the brush border of the small intestine (Mantei et al. 1988; Sahi 1994a). The enzyme is essential for the digestion of lactose and ensures that the monosaccharides glucose and galactose can be absorbed in the blood stream. The production of LPH is high in infants, however, the LPH level decrease with age and in approximately 70% of the world population the enzyme production is down regulated. The production of LPH is genetically determined and has developed from a strong positive natural selection the last 10 000 years, when cattle was first domesticated (Itan et al. 2010). The domestication started in the Middle East and was spread to Europe and parts of Africa, and there are high correlations between lactase persistence and a history of dairying (Itan et al. 2010). The production of the LPH enzyme is determined by a gene called LCT, which is regulated by a promoter gene, MCM6, where many of the mutations associated with lactase persistence is found (Tishkoff et al. 2007; Ranciaro et al. 2014). Different theories have evoked and discussed to describe the continuous production of LPH throughout lifetime. The high correlation between lactase persistence and geographic regions with a high prevalence of dairying has mainly been explained by a positive selected mutation in genes associated with lactase persistence. Another theory suggests that the calcium and vitamin D present in milk could prevent populations from diseases such as rickets, and therefore the lactose persistence developed (Flatz and Rotthauwe 1973; Itan et al. 2010). A recent study, made by Sverrisdóttir et al. (2014) investigated a specific mutation associated with lactase persistence (LCT-13,910*T) and concluded that calcium and vitamin D in the milk were not the reason for the positive natural selection, furthermore the study concluded that other “evolutionary selective pressures” such as starvation have affected the high prevalence of lactase persistence in Europe (Sverrisdóttir et al. 2014).

Different terminologies, such as hypolactasia, lactose malabsorption, maldigestion and lactose intolerance are used in relation to a down-regulated, and thereby, a low production of LPH in the small intestine. In this thesis, lactose intolerance is used as a term referring to an insufficient production of the
enzyme, which gives rise to problems such as abdominal pain, flatulence and diarrhea (Sahi 1994b). The abdominal problems are results of lactose not being hydrolyzed in the small intestine, which mean that lactose continues down to the large intestine. Bacteria present in the large intestine will hydrolyze the lactose and ferment the monosaccharides, which lead to production of gas and short chain fatty acids. Furthermore, water will be drawn into the large intestine from the blood by osmosis to balance the high concentration of lactose, which may result in diarrhea.

3.2 Production of lactose-hydrolyzed milk

Lactose-hydrolyzed UHT treated milk can be produced in different ways, however, the production generally includes one or more filtration steps (ultrafiltration, nanofiltration, osmosis) and an enzymatic hydrolysation (Jelen and Tossavainen 2003; Harju et al. 2012). The lactose can be hydrolyzed before (pre hydrolysis) or after (post hydrolysis) the applied heat treatment, and the milk can be direct or indirect heat treated. In the present study the milk was posthydrolyzed, and direct or indirect UHT heat treated. The production of lactose-hydrolyzed UHT milk is similar to the production of conventional UHT milk (chapter 2:figure 5), however, two additional steps are included. The first additional step is ultra-filtration (UF) in order to separate the lactose, water and minerals (permeate) from the proteins and fat (retentate), the permeate is subsequently nanofiltrated (NF) to separate the lactose (permeate) from the water and minerals (retentate). The NF retentate is then added to the UF retentate again and milk with 40% reduced level of lactose is obtained. A part of the lactose is removed from the lactose-hydrolyzed milk to obtain the same sweetness of the milk as perceived in conventional milk. The milk is then heat treated (UHT), followed by the second additional step including the enzymatic hydrolysis where the enzyme is added to the milk aseptically to hydrolyze the remaining lactose.

The hydrolysation of lactose is dependent on production time and capacity, enzyme dose and temperature during the hydrolysis. In the posthydrolyzed approach the dosing of enzyme is low since the hydrolysis take place in the package during transportation to the suppliers. However, the aseptic enzyme is more expensive than the non-aseptic enzyme used in the prehydrolyzed milk. In the prehydrolyzed milk the enzyme is added batch wise, and a high enzyme dose is required to increase hydrolysation rate to avoid a long storage time before the heat treatment (Harju et al. 2012).
3.3 β-galactosidase

The enzyme used to hydrolyze lactose in milk is β-galactosidase (lactase), which has a high specificity for O-glycosyl bonds in lactose. In addition to the natural production of LPH in the small intestine of mammals, β-galactosidase is found in bacteria, yeast and fungi, which are used for production of commercial β-galactosidase. The different sources of β-galactosidase have different pH optima and can thereby be used in different dairy products such as fermented products with low pH and in milk with a neutral pH. The enzymes most applied in the industry are from *Kluyveromyces fragilis* and *Aspergillus niger* since their pH optimum are pH 6.5-7.0 and pH 2.5-4.0, respectively, which is highly suitable for dairy products (Mahoney 2003).

The enzymes are often intracellular, and the cells must therefore be disrupted to obtain the β-galactosidase. When the cell is disrupted other enzymes may also be included in the β-galactosidase preparation, which for that reason must be purified. To purify the enzyme preparation for β-galactosidase gel filtration, ion exchange chromatography and affinity chromatography can be used. However, the preparation never obtains 100% purity and the enzyme preparation therefore contains impurities, such as arylsulfatase and proteases, which can affect the dairy product quality (Mittal et al. 1991; De et al. 2007). Mittal et al. (1991) investigated different commercial lactase and observed that higher lactase purity decreased the proteolytic activity in the milk during storage, and that the price of the enzyme correlated with the purity and thereby the quality of the milk. However, there exist only a limited number of published studies about the quality of different β-galactosidase enzymes.
Chapter 3. Lactose-hydrolyzed milk
Chapter 4. Analytical methods

4.1 Dynamic headspace sampling gas chromatography-mass spectrometry

4.1.1 Analysis of volatile compounds by dynamic headspace sampling gas chromatography-mass spectrometry

In milk, more than 400 volatile compounds belonging to different chemical classes have been identified (Badings et al. 1981). Some of the volatile compounds are present in very low concentrations and need to be separated and isolated prior to the analysis by gas chromatography-mass spectrometry (GC-MS) (Señorans et al. 1996). Dynamic headspace sampling (DHS) is a solvent-free extraction method used in combination with GC-MS. It is desirable that the composition of the volatiles is not altered by the extraction method. However, this is difficult to achieve and often the method should be optimized according to the most important compounds in the milk for the specific study, which is discussed in chapter 4.1.2 as well as in paper 1. DHS-GC-MS (Figure 7) is a holistic extraction and analysis method for volatile compounds and includes an extraction, desorption, separation and an identification step. In the extraction step, an inert gas is purged through or above the sample to collect and transport the volatiles to trap the volatiles on an adsorbent trap. Subsequently, the volatile compounds are released from the trap by thermal desorption and transferred directly to a GC-column to be separated by their volatility (Werkhoff and Bretschneider 1987; Johns et al. 2005). When the migrated compounds have reached the end of the GC-column they enter a detector, and often it is a mass spectrometry detector that identifies the compounds.

Figure 7. Dynamic headspace sampling coupled to adsorbent traps, autosampler, coldtrap, gas chromatograph and mass spectrometer
An alternative, solvent-free method to extract volatile compounds is solid phase micro-extraction (SPME), which is an often used method. Both DHS and headspace SPME require controlled extraction conditions such as temperature, sample volume and extraction time. The sample volume and extraction time required can be considerably lower in headspace SPME than in DHS. Furthermore, SPME does not require a desorption step as DHS, which makes SPME a time-effective and inexpensive extraction method. For both extraction methods, the concentration and number of extracted compounds is dependent on extraction temperature, and increasing temperature increases the concentration of the extracted compounds (Contarini and Leardi 1994; Vazquez-Landaverde et al. 2005), although the volatiles can be extracted at room temperature. Similar to DHS, where different adsorbent traps materials are applied to collect different volatiles, SPME also has different fiber materials with different affinity for volatile compounds.

DHS-GC-MS is an attractive method when the compounds in the sample under investigation are unknown prior to the analysis, and DHS-GC-MS has been widely used for analysis of volatiles in a range of different food matrixes (Contarini et al. 1997; Jensen et al. 2011; Bach et al. 2012; Reboredo-Rodríguez et al. 2012; Nightingale et al. 2012; Jansson et al. 2014). However, since SPME is considered an inexpensive and time effective extraction method, the outcome of SPME and DHS-GC-MS has been compared for various food products (Elmore et al. 1997; Marsili 1999; Contarini and Povolo 2002; Povolo and Contarini 2003; Kanavouras et al. 2005). Contarini et al. (2002) compared the two extraction methods for discrimination of pasteurized, UHT and sterilized heated whole milk. In the purge and trap method room tempered milk was purged for one hour with nitrogen (Contarini and Povolo 2002). In the SPME method, milk heated to 45 °C was extracted for 30 minutes during stirring. The conclusion from the study was that the two extraction methods could discriminate the milk types by 11 volatiles, although the type and concentrations of the volatiles differed between the methods. However, a more comparable evaluation of the extraction methods would have been obtained if the same extraction temperature had been applied for both methods. Povolo and Contarini (2003) analyzed butter (45 °C) with DHS and SPME extracting the volatiles from the butter for 1 hour and 30 minutes, respectively. The number of volatiles extracted with DHS was two times higher as for SPME, which could be ascribed to the larger surface area of the adsorbent trap (Tenax TA) compared to the fiber used in the SPME method. Marsili (1999) investigated specific lipid oxidation aldehydes in milk with DHS and SPME. The milk was heated to 45 °C followed by the extraction by DHS or SPME. After the DHS extraction the adsorbent trap
was dry-purged to remove water vapor, followed by desorption and analysis. A lower variation between the replicates and a higher linearity coefficient of the calibration curves for the aldehydes were observed for the SPME method compared to the DHS. Thus, a higher precision was observed for the SPME samples compared to the DHS (Marsili 1999). It could be considered if the dry-purging of the tubes in the DHS extraction affected the precision of the method.

It may be concluded that DHS and SPME are somewhat comparable in reproducibility and sensitivity. However, DHS may be the preferred method for trace analysis due to larger surface area of the adsorbent tube which will extract a higher amount and a higher number of different volatiles than SPME (Elmore et al. 1997; Povolo and Contarini 2003; Kanavouras et al. 2005). In addition, the volatile compounds extracted with DHS and SPME may vary between the two techniques.

**4.1.2 Optimization of DHS-GC-MS method**

It is desirable that the DHS-GC-MS method used for milk analysis is optimized to detect as many volatiles in the milk as possible. During the extraction step, factors such as temperature, gas flow, time and sampling volume is important for the amount and number of volatiles collected (Vallejo-cordoba and Nakai 1993; Contarini and Leardi 1994). The adsorbent tube material also has considerable impact on which volatiles that are collected in the extraction step. In the desorption step the coldtrap material may be considered as well as the cryofocusing and desorption temperature which may influence the evaporation and peak shape of the volatiles. It is desirable to avoid water in both the extraction and desorption step, consequently the adsorbent tubes may be back-purged, and a two-stage desorption may be applied. In the separation step the temperature gradient is often optimized to increase the separation of the compounds with similar volatility as well as to shorten the total analyzing time. In the identification step selected ion monitoring (SIM) can be applied to search for specific volatile compounds. SIM may preferable be used for identification of volatile compounds present at very low concentrations that are of particular interest. The principle for SIM is that specific ions are selected for identification of the specific compounds which increases the specificity for the particular compound and decrease involvement of unwanted compounds in the peaks, consequently the identification and quantification become more certain.
4.1.2.1 Optimization of extraction step of the volatile compounds in milk

In the present study an extraction temperature of 20 °C was applied so that the volatile profile was comparable with the sensory descriptive analysis of the same milk served at 14 °C. Previous studies applying headspace extraction to analyze milk volatiles extract at higher temperatures e.g. 45 °C to increase the concentrations and number of identified volatiles (Señorans et al. 1996; Valero et al. 2001; Vazquez-Landaverde et al. 2005; Vazquez-Landaverde et al. 2006). Valero et al (2001) extracted UHT milk at 45 °C and found 41 and 55 volatile compounds in whole and skim milk, respectively. Contarini et al. (1997) extracted UHT milk with DHS at room temperature and identified 11 volatile compounds which is considerably lower than the 28 volatile compounds identified in the present study (table 1). The extraction time, sample volume and gas flow were also optimized in order to increase the concentration of the detected volatiles (paper 1).

Different adsorbent tube materials were investigated and the porous polymer Tenax TA tubes were found to be the adsorbent material that extracted highest amount of compounds, with high affinity for most of the volatile compounds in the milk. In addition, Tenax TA has a low affinity for water (Helmig and Vierling 1995), which may disturb the extraction of the volatile compounds in the milk. After extraction, the adsorbent traps were back-purged with nitrogen in order to remove water that potentially had been absorbed in the traps during the extraction. However, the water did not appear to interact with the volatiles and the back-purging resulted in a decreased concentration of volatiles and was therefore not included in the extraction method.

4.1.2.2 Optimization of the desorption step of the volatile compounds in milk

The cryofocusing was investigated and was changed between -10 °C, 1 °C and 30 °C where a higher temperature theoretically ought to decrease the condensed water in the coldtrap. However, 1 °C was found to be the optimal coldtrap temperature.

Desorption of the volatiles was also improved in relation to water. A two-stage tube desorption was evaluated to evaporate water at 100 °C in a first stage and subsequently desorb the volatiles in a second stage by increasing the temperature to 250 °C. However, this was found not to be effective for milk as
the volatiles were evaporated together with the water in the first step and thereby lost for identification.

4.1.2.3 Optimization of the analyzing and identification of the volatile compounds in milk

The temperature gradient was optimized to separate the volatile compounds, especially the highly volatile compounds eluting in the first part of the chromatogram. The analyzing time could also be shortened by increasing the temperature gradient in the chromatogram where no volatile compounds were present.

The volatiles were analyzed in total ion chromatogram (TIC) mode, followed by identification. Subsequently, SIM was successfully used in the present study to analyze volatile compounds that was considered important for the development of the Maillard reaction in the milk. In table 1 all the identified compounds are listed as well as the compounds analyzed with SIM mode. In addition, the specific ions that were used for quantification of the specific compounds are presented.
Table 1. Overview of the volatile compounds identified with GC-MS, the retention time (Rt) and the ions used for quantification in SIM mode in the present study (paper 2).

<table>
<thead>
<tr>
<th>Rt (min)</th>
<th>Volatile compound</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.84</td>
<td>3,3-dimethylhexane</td>
<td></td>
</tr>
<tr>
<td>6.18</td>
<td>2-methylfuran</td>
<td>82</td>
</tr>
<tr>
<td>6.87</td>
<td>2-butanone</td>
<td></td>
</tr>
<tr>
<td>7.05</td>
<td>2-methylbutanal</td>
<td>86</td>
</tr>
<tr>
<td>8.22</td>
<td>2-ethylfuran</td>
<td>81</td>
</tr>
<tr>
<td>9.20</td>
<td>2-pentanone</td>
<td></td>
</tr>
<tr>
<td>10.12</td>
<td>Decane</td>
<td></td>
</tr>
<tr>
<td>12.14</td>
<td>Toluene</td>
<td></td>
</tr>
<tr>
<td>14.48</td>
<td>Dimethyl disulfide</td>
<td></td>
</tr>
<tr>
<td>15.47</td>
<td>2-hexanone</td>
<td></td>
</tr>
<tr>
<td>15.50</td>
<td>Hexanal</td>
<td>82</td>
</tr>
<tr>
<td>27.37</td>
<td>2-heptanone</td>
<td></td>
</tr>
<tr>
<td>27.38</td>
<td>Heptanal</td>
<td>70</td>
</tr>
<tr>
<td>30.48</td>
<td>Dodecane</td>
<td></td>
</tr>
<tr>
<td>32.69</td>
<td>2-pentylfuran</td>
<td>95</td>
</tr>
<tr>
<td>34.84</td>
<td>Styrene</td>
<td></td>
</tr>
<tr>
<td>36.75</td>
<td>2-octanone</td>
<td></td>
</tr>
<tr>
<td>36.94</td>
<td>Octanal</td>
<td></td>
</tr>
<tr>
<td>39.07</td>
<td>6-methyl-5-heptene-2-one</td>
<td></td>
</tr>
<tr>
<td>40.12</td>
<td>Dimethyl trisulfide</td>
<td></td>
</tr>
<tr>
<td>40.75</td>
<td>2-nonanone</td>
<td></td>
</tr>
<tr>
<td>40.95</td>
<td>Nonanal</td>
<td></td>
</tr>
<tr>
<td>41.14</td>
<td>Tetradecane</td>
<td></td>
</tr>
<tr>
<td>42.91</td>
<td>Furfural</td>
<td></td>
</tr>
<tr>
<td>43.42</td>
<td>Decanal</td>
<td></td>
</tr>
<tr>
<td>45.05</td>
<td>Benzaldehyde</td>
<td></td>
</tr>
<tr>
<td>45.25</td>
<td>2-undecanone</td>
<td>170</td>
</tr>
<tr>
<td>48.90</td>
<td>6,10-dimethyl-5-undecadiene-2-one</td>
<td></td>
</tr>
</tbody>
</table>
4.2 $^1$H NMR spectroscopy

$^1$H NMR spectroscopy is a nondestructive technique without extensive sample preparation that is used to analyze the presence and molecular structure of organic molecules and metabolites, such as carbohydrates, organic acids, vitamins, amino acids and aromatic compounds present in fluids. $^1$H NMR spectroscopy can be applied on complex mixtures, however a disadvantage with $^1$H NMR spectroscopy is that highly abundant compounds in a sample may overlap with less abundant compounds with similar chemical shift. This means that the dynamic range is limited. In milk, removing proteins and fat prior to the analysis by precipitation, filtration or ultracentrifugation will improve the resolution and detection of low-abundant metabolites.

$^1$H NMR spectroscopy has been applied in studies investigating metabolites in milk (Hu et al. 2007; Klein et al. 2010; Sundekilde et al. 2013b). Sundekilde et al. (2013a) assigned a total of 40 milk metabolites including amino acids, organic acids and carbohydrates (Sundekilde et al. 2013a). In addition, $^1$H NMR spectroscopy has been applied to analyze lactose-hydrolyzed milk (Monakhova et al. 2012; Jansson et al. 2014). Monakhova et al (2012) investigated the lactose concentration in lactose-hydrolyzed milk with 1D and 2D J-resolved NMR spectroscopy, and the technique could detect a lactose level as low as 0.05 g/L with high accuracy. The level of the acyclic form of reducing carbohydrates is highly important for the extent of reactions taking place in the milk, especially in relation to Maillard reactions. By using a combination of [1-$^{13}$C]-labeled aldohexoses and $^{13}$C NMR spectroscopy the proportion of acyclic and cyclic tautomers of different aldohexoses has been determined (Zhu et al. 2001). Zhu et al. (2001) found a higher percentage of acyclic form for galactose than for glucose, which support the fact that galactose is more reactive compared with glucose (Naranjo et al. 2013).

4.3 Methods for characterization of proteins

4.3.1 RP HPLC MS analysis of intact and hydrolyzed proteins

HPLC is used to separate and analyze non-volatile compounds including proteins. The HPLC system consists of a stationary and mobile phase and high pressure is applied to force the solvent through the system (Harris 2007). In reversed phase chromatography the solvent is polar and the stationary phase (the column) nonpolar. Proteins will be retained and eluted from the column according to their hydrophobicity by using a solvent with increasing organic
content. Bovine milk proteins have previously been analyzed using reverse phase chromatography (Bonfatti et al. 2008; Jensen et al. 2012) where the elution order of the proteins was found as follows: glycosylated fraction of κ-CN and glycoproteins (presume to comprise all major milk proteins), followed by unglycosylated fraction of κ-CN, αS2-CN, αS1-CN, β-CN and finally the major whey proteins β-LG and α-LA. Ultraviolet (UV) detection is the most commonly applied method to detect the separated analytes (Harris 2007). In the present study the eluted proteins were detected with UV absorbance at 214 nm measuring the sum of amide bonds with signal intensity mainly proportional to the analyte concentration. The separated and detected proteins may subsequently be characterized by MS analysis (Steen and Mann 2004; El-Aneel et al. 2009). The chromatographic profile of the conventional UHT milk is presented in Figure 8.

![Image](image.jpg)

**Figure 8.** Protein profile of conventional UHT milk detected by UV absorbance at 214 nm during storage (paper 3).

HPLC can be used for analysis of proteins and peptides and to elucidate proteolysis as well as lactosylation of proteins in milk (Le et al. 2006; Czerwenka et al. 2006; Wedholm et al. 2008). As an example Wedholm et al (2008) investigated peptides in milk and related the cleavage sites of the peptides with the type of indigenous milk enzymes present in the milk.
4.3.2 RP HPLC MS for analysis of furosine

RP HPLC MS is a convenient and rapid method that can be applied to analyze furosine in milk. In the present study furosine was analyzed by RP HPLC MS with isocratic elution (0.06 M sodium acetate buffer) using a Supelco supercosil LC-8 column, and detected at 280 nm as described in ISO 18329:2004 (International Organization for Standardization and International Dairy Federation 2004) and in Paper 2. The advantage with isocratic conditions is that the furosine peak elutes early and thereby the analysis time is reduced compared with gradient elution (Serrano et al. 2002). However, it has been suggested that analyzing furosine with gradient elution conditions decrease the involvement of other compounds and thus increase the purity of the furosine peak (Delgado et al. 1992). Prior to the HPLC analysis the proteins are hydrolyzed with acid, which may affect the concentration of Amadori product that is converted to furosine considerably. Serrano et al. (2002) investigated the effect of 6M, 8M and 10M acid concentrations on furosine concentration in milk powder. The study revealed that the furosine concentration increased with increasing acid concentration, thus a better relation between the Amadori product and furosine was obtained. In addition to RP HPLC MS, furosine may be determined by ion-exchange chromatography, gas chromatography and capillary electrophoresis (Serrano et al. 2002).

4.3.3 Fluorescamine assay for analysis of free amino terminals

Fluorescamine assay is a rapid method to analyze free amino terminals (primary amino groups) situated on amino acids, peptides and proteins (Udenfriend et al. 1972). The non-fluorescent fluorescamine reacts rapidly with the primary amines generating stable fluorophors that can be analyzed by its excitation and emission wavelengths of 400 nm and 485 nm (Beeby 1980). In the present study, the free amino terminals revealed information about the proteolytic activity in the milk (paper 2). However, the free amino terminals can also be used to determine the level of lactosylation which is indicated by a decrease in the level of free amino terminals (Dalsgaard et al. 2007).
4.4 Sensory descriptive analysis

Sensory descriptive analysis was used to define the sensory characteristics of the milk in the present study. The results from the analysis is presented and discussed in paper 4. Descriptive analysis is a sophisticated and useful tool to describe and differentiate products according to their sensory characteristics. In short, descriptive analysis can be divided into three steps; discussion, training, and analysis. In the first step in the present study the panelists were presented for reference samples representing sensory attributes commonly associated with storage related off-flavors in milk as well as extreme milk samples from the sample set (paper 4). The panelists agreed upon a list of sensory attributes that could be used to differentiate the milk samples. In sensory descriptive analysis it is important that the attributes used do not correlate, and that they are as descriptive as possible, to facilitate the evaluation for the panelists (Stone et al. 1974). The attributes are divided into different sensory impressions: appearance, aroma, flavor, taste and touch. Appearance is evaluated by sight, aroma is perceived orthonasally, flavor is perceived retronasally (Rozin 1982), taste is determined by taste buds present on the tongue and touch is perceived using the somatosensory system (Lawless and Heymann 2010). The attributes used to characterize the milk in paper 4 is presented in table 2. The second step, was training of the panelists. Training is conducted in order to improve the panelists performance in form of their ability to differentiate between samples, reproducibility and consistency. In this step, the panelists receive feedback on their performance after each training session. Finally, in the third step, the actual sensory descriptive analysis was conducted (Lawless and Hildegarde 2010).

Table 2. Overview of attributes used to differentiate between conventional, filtered and lactose-hydrolyzed UHT milk

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Popcorn aroma</td>
<td>Freshly made popcorn</td>
</tr>
<tr>
<td>Boiled aroma</td>
<td>Boiled/heated milk, like rice porridge</td>
</tr>
<tr>
<td>Stale aroma</td>
<td>Staled stored aroma</td>
</tr>
<tr>
<td>Color saturation</td>
<td>Saturation of white color</td>
</tr>
<tr>
<td>Creamy flavor</td>
<td>The flavor of double cream</td>
</tr>
<tr>
<td>Boiled milk flavor</td>
<td>Rice porridge</td>
</tr>
<tr>
<td>Popcorn flavor</td>
<td>Popcorn/burned</td>
</tr>
<tr>
<td>Caramel flavor</td>
<td>Toffee, aromatic sweet</td>
</tr>
<tr>
<td>Stale flavor</td>
<td>Stale/stored flavor, open milk in fridge for days</td>
</tr>
<tr>
<td>Sweet taste</td>
<td>Pure sweet taste, appear at once</td>
</tr>
<tr>
<td>Bitter taste</td>
<td>The basic bitter taste</td>
</tr>
<tr>
<td>Fatty mouth feel</td>
<td>Creamy mouth feel</td>
</tr>
</tbody>
</table>
Chapter 5. The Maillard reaction

In this chapter the Maillard reaction will be discussed including the results obtained in the present project in relation to the Maillard reaction. Dynamic headspace sampling-gas chromatography-mass spectrometry (DHS-GC-MS) was used to analyze volatile compounds (chapter 4.1) and reverse-phase high pressure liquid chromatography mass spectrometry (RP-HPLC MS) was used to analyze furosine and glycated proteins present in the milk (chapter 4.3). In addition, proton nuclear magnetic resonance spectroscopy (¹H NMR) was used to analyze carbohydrates, formate and acetate in the milk (chapter 4.2).

5.1 Maillard reaction theory

The Maillard reaction was first observed in 1912 by Louis-Camille Maillard (Maillard 1916) and the complex reaction has been widely reviewed ever since, but still its products are not yet fully characterized. In food products the Maillard reaction is highly relevant due to the formation of compounds contributing to changes in aroma, flavor, taste and color. In milk these changes are unwanted as they decrease the acceptance and shelf-life of the milk. In addition, the Maillard reaction has a pronounced effect on the nutritive value in the food. Increased lactosylation of proteins decrease the level of available lysine, consequently, a nutritive loss is observed in heated and stored milk (Burvall et al. 1977). Furthermore, in humans, advanced glycation end-products (AGEs) formed in the Maillard reaction cascade have been associated with Alzheimer disease and diabetes (Smith et al. 1994; Thorpe and Baynes 1996). Hodge (1953) divided the Maillard reaction into an early, advanced and final stage (Hodge 1953). This three-stage classification is still accepted and will be used to describe the Maillard reaction in this chapter.

The early stage Maillard reaction in milk involves a condensation of an α-hydroxy group situated on a reducing carbohydrate and a free amino group on a protein or peptide (mainly ε-amino group of lysine). The condensed compounds are unstable (also called Schiff base) and are rapidly rearranged to more stable Amadori products (Figure 9). The Amadori products that are derived from lactose, glucose and galactose are ε-lactulosyllysine, ε-fructoselysine and ε-tagatoselysine respectively (Van Renterghem and De Block 1996; Evangelisti et al. 1999; Erbersdobler and Faist 2001; Mendoza et al. 2005; Fox 2009). Furthermore, acid hydrolysis and heating of the Amadori products result in furosine, which is used as heat-load indicator in milk products.
However, due to an incomplete conversion of Amadori products to furosine, furosine cannot be used as a direct measure of the glycated protein (Erbersdobler and Somoza 2007; Meltretter et al. 2009).

Figure 9. Condensation of aldose with amino group leading to Amadori product via Schiff base and Amadori rearrangement.

In the advanced stage, the Amadori products are degraded via enolation (Yaylayan and Huyghues-Despointes 1994; Kokkinidou and Peterson 2014). The Amadori products are in equilibrium with 2,3-eneaminol and 1,2-eneaminol and depending on pH, the two eneaminols give rise to different, highly reactive, dicarbonyl compounds. In milk the pH is approximately 6.6, which favors the 2,3-eneaminol formation and generate 1-deoxyosone by amino elimination. The 1,2-eneaminol pathway, at acidic pH, favors the formation of 3-deoxyosone by water elimination, as well as other different degradation products, such as furfural and hydroxymethylfurfural. The deoxyosone formation is followed by fission degradation. Its products are pyruvaldehyde, methylglyoxal, diacetyl, galactose and reductones such as formic and acetic acid (Ledl and Schleicher 1990; Weenen 1998; Van Boekel 1998; Brands and van Boekel 2001; Davidek et al. 2002; Davidek et al. 2006; Smuda and Glomb 2013). Methylglyoxal is a reactive α-dicarbonyl that has received a lot of attention due to its contribution to flavor in food and its formation of AGEs. Carbonyls can be formed from carbohydrate degradation, from the Schiff base in the early stage of the Maillard reaction and from the deoxyosones (Wang and Ho 2012). In presence of amino acids, the deoxyosones and other carbonyls react with the amino acids which undergo a decarboxylating transamination resulting in loss of a carbon atom and formation of Strecker degradation compounds. During the formation of Strecker compounds, flavor, carbon dioxide as well as formation of new carbonyls are released (Hofmann et al. 2000; Hofmann and Schieberle 2000; Belitz et al. 2001) (Figure 10). The Strecker compounds contribute to flavor, but can also react further, via aldol condensation, to give different dimeric products, which
contribute to odor in food. In table 3 an overview of the amino acids contributing to Strecker degradation compounds is shown.

![Reaction Diagram](image)

**Figure 10. Formation of Strecker degradation products.**

**Table 3. Overview of amino acid involvement in the formation of Strecker degradation aldehydes and secondary reaction products** (Whitfield and Mottram 1992; Pripis-Nicolau et al. 2000; Limacher et al. 2008).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Strecker aldehydes</th>
<th>Secondary reaction products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Acetaldehyde</td>
<td>2-Methylfuran, 2-pentylfuran</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Acetaldehyde</td>
<td>Hydrogen sulfide, methanethiol</td>
</tr>
<tr>
<td>Glycine</td>
<td>Formaldehyde</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2-Methylbutanal</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>3-Methylbutanal</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>Methional</td>
<td>Dimethylsulfide, Dimethyltrisulfide</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Benzaldehyde, phenylacetaldehyde</td>
<td>Furan, 2-methylfuran, pyrazine</td>
</tr>
<tr>
<td>Serine</td>
<td>2-Hydroxyethanal</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>Lactaldehyde</td>
<td>2-Methylfuran</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2-(p-Hydroxyphenyl)ethanal</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>2-Methylpropanal</td>
<td></td>
</tr>
</tbody>
</table>

In the final stage, the aldoses are condensed, the aldehydes and amines are polymerized and heterocyclic nitrogen compounds are formed, resulting in high-molecular-weight compounds, colored products e.g. melanoidins (Hodge 1953; Brands et al. 2002).

In addition to the more general Maillard reaction cascade described by Hodge (1953), Yaylayan 1997 have added reactions to the model. Yaylayan (1997) states that carbohydrates, proteins and amino acids as well as Amadori products can generate fragmentation pools that react together or independently, forming different degradation products, which are further included in reactions to form compounds giving flavor and color (Yaylayan 1997). However, the carbohydrate fragmentation is favored by the presence of amino acids.
5.2. Maillard reactions in conventional and lactose-hydrolyzed UHT milk

The present study aims to analyze specific Maillard reaction products that could be important to off-flavor in lactose-hydrolyzed UHT milk, which will be discussed in this chapter.

$^1$H NMR spectroscopy was applied to analyze carbohydrates present in the milk in order to investigate if the Maillard reaction changes the level of carbohydrates in the milk (paper 3). The anomers of glucose at 5.23 ppm and 4.63 ppm, galactose at 5.26 ppm, and lactose at 5.23 ppm were quantified. No pronounced change was observed in the levels of carbohydrates which most probably indicate that the carbohydrates are not a limiting factor in the reaction. A decrease could be expected at least in the lactose-hydrolyzed milk due to the severe Maillard reaction occurring. However, a possible explanation for the observation is that in order to observe carbohydrate degradation severe heat treatment during a long time is necessary (Van Boekel 1998). $^1$H NMR spectroscopy did however reveal a decrease in the level of glucose and galactose in the lactose-hydrolyzed milk between storage day 240 and 270 (paper 3). As the same pattern was observed for the free amino acids present in the lactose-hydrolyzed milk (paper 3:table 2:figure 1), this could indicate initiation of carbohydrates and protein condensation and polymerization, and formation of melanoidins.

5.2.1 Early stage Maillard reaction

Furosine, which is an indicator for the early stage of the Maillard reaction, was analyzed by HPLC (paper 2). The level of furosine in milk stored for 60 days was higher in the conventional than in the lactose-hydrolyzed UHT milk (Figure 11). This indicates that the Amadori products were formed during the heat treatment since the carbohydrate concentration at the time point of heat treatment is higher in the conventional milk than in the lactose-hydrolyzed milk. This suggests that the level of carbohydrates is important for the Amadori product formation and that the carbohydrates may have been a limiting factor for the formation of these in the lactose-hydrolyzed milk. Furosine increased in both conventional and lactose-hydrolyzed milk during storage. However, the formation rate of furosine in the lactose-hydrolyzed milk was significantly higher in the lactose-hydrolyzed than in the conventional milk throughout the storage time. This result indicates that the type of sugar has major impact on the formation of Amadori products during storage, and that glucose and galactose
condense with proteins to a higher degree than lactose. This finding elaborates with previous studies on sugar types in relation with Maillard reaction rate (Kato et al. 1988; Naranjo et al. 2013). The Amadori products formed in the milk is highly dependent on the condensation between the reducing carbohydrates and the proteins in the milk, which is affected by the configuration, glass transition temperature and the reactivity of each carbohydrate as discussed in chapter 1. Naranjo et al. (2013) heated glucose, galactose and lactose with casein and stored the mixtures at 37 °C for several hours and analyzed the loss of carbohydrates continuously during the storage time. The rate constants (mol h⁻¹) were calculated from the curves obtained for the decrease of each carbohydrate as percentage of the total carbohydrate concentration in an unheated control sample. Naranjo et al. (2013) found highest rate constant for galactose, followed by glucose and finally lactose, which indicated that galactose had the highest reactivity with casein compared with glucose and lactose. This finding in combination with the increased Tg for galactose and glucose compared to lactose may describe the increased level of furosine observed in figure 11. Kato et al. (1988) compared condensation of ovalbumin with glucose and lactose and found similar condensation between the two carbohydrates with ovalbumin, although the amount of free lysine and Schiff bases were lower and the amount of furosine was higher in the glucose samples compared to the lactose samples. These results suggested a faster and easier Amadori rearrangement in glucose-containing samples.

Tossavainen and Kallioinen (2008) analyzed furosine in lactose-hydrolyzed and conventional UHT milk during a three month storage period at 5, 22, 30 and 45 °C. The level of furosine was highly dependent on the storage time and temperature, where the level of furosine increased with increasing storage temperature in the conventional and lactose-hydrolyzed UHT milk. The hydrolysis of lactose had a significant effect on the level of furosine, and for the milk stored at 22 °C approximately 270 mg/100 g protein was observed in the lactose-hydrolyzed UHT milk, compared with approximately 90 mg/100 g furosine in the conventional UHT milk (Tossavainen and Kallioinen 2008). Messia et al. (2007) analyzed furosine in conventional and lactose-hydrolyzed UHT milk during a 4 month storage period at 20 °C. The level of furosine in indirect heated lactose-hydrolyzed UHT milk reached 562 mg/100 g protein after 4 months of storage while the furosine content was 310 mg/100 g protein in indirect heated conventional UHT milk. Furthermore, Messia et al. (2007) investigated how direct UHT treatment affected the level of furosine in the lactose-hydrolyzed and conventional milk. The level of furosine decreased in
both milk types, and after 4 months of storage the furosine level was 480 and 126 mg/100 g protein in the lactose-hydrolyzed and conventional milk, respectively (Messia et al. 2007).

The results obtained from Tossavainen and Kallioinen (2008) and Messia et al. (2007) are comparable with the results obtained in this study (paper 2). Shown in figure 11 the level of furosine in the lactose-hydrolyzed milk after 3-4 months of storage was 400-500mg/100 g protein and the level of furosine in the conventional milk remained somewhat stable at 250mg/100 g protein. However, the furosine levels are higher in the present study compared to Tossavainen and Kallioinen (2008) and Messia et al. (2007), and no effect of type of heat treatment could be observed for the lactose-hydrolyzed UHT milk. These differences could probably be explained by different heat treatment parameters applied to the milk.

![Figure 11. Formation of furosine in conventional and lactose-hydrolyzed UHT milk.](image)

Glycated proteins were analyzed with RP HPLC MS in this study, as presented in paper 3. The existence of glycated products were confirmed in all milk types at all storage times (14-270 days) as at least one or more of the identified major identified proteins ($\kappa$-CN, $\alpha_{\text{S2}}$-CN, $\alpha_{\text{S1}}$-CN, $\beta$-CN and $\beta$-LG) had mass additions of +162 Da for one hexose, i.e. glucose or galactose, or +324 Da for lactose itself, as well as multiple hexoses (data not shown). By the method applied in the present study, the glycated proteins had similar elution time in the chromatographic profile which contributed to difficulties to integrate or quantify a single glycated protein. To quantify the glycated proteins, further separation of the proteins would have been necessary.
Moreover, a total pool of glycated (as a result of initial Maillard reaction) and glycosylated (formed during the biosynthesis of the proteins in the lactating epithelial cells) proteins were observed in the chromatographic profile as the glycoprotein, obtained using UV absorbance (figure 8). The level of the glycoprotein fraction significantly increased in all milk types during storage, and significantly more in the indirect lactose-hydrolyzed UHT milk compared to the other milk types (paper 3:table 2). The slightly higher level of glycoprotein in the indirect lactose-hydrolyzed UHT milk indicates that sugar type affect the glycated proteins in the milk, furthermore, the lower heat load from the direct UHT treatment seems to affect the level of glycoprotein. The glycoprotein comprised both the fraction of the glycated milk proteins, as well as the O-glycosylated κ-CN naturally present in the milk. It could be expected that the lactose-hydrolyzed milk had a higher level of glycoprotein compared to the conventional milk due to the higher levels of furosine observed in paper 2. Carulli et al. (2011) investigated reactivity of lactose, glucose and galactose with α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) and observed increased glycation rate for glucose and galactose including an increased level of glycated α-LA, compared to lactose (Carulli et al. 2011).

5.2.2 Advanced stage Maillard reaction

Furfural was identified with DHS-GC-MS and the content remained unchanged during storage, consequently it was concluded that furfural was formed during the heat treatment, and that no formation was taking place during storage (paper 2:figure 5). Furfural is formed via the 3-deoxyosone pathway by reduction of water. A small concentration of furfural has been detected in milk (Berg 1993), and it increased with increased severity of the heat treatment applied, which supports that heat is the main cause for the formation of furfural in milk. Furfural can also be formed from carbohydrate degradation (Ferrer et al. 2002), however, this did not seem to affect the level of furfural in the present study, probably due to minimal carbohydrate degradation during storage at room temperature revealed from the 1H NMR analysis (paper 3:figure 2).

Acetic acid and formic acid were analyzed by 1H NMR spectroscopy. The concentration of acetic (paper 2:figure 9) and formic (paper 3:table 2) acid increased during storage for all the milk types and the changes were not significantly different between the milk types. However, the initial level of formic acid was considerably higher in conventional milk than in the lactose-hydrolyzed and filtered milk; thus, a clear effect of the level of carbohydrates present in the milk during heat treatment was seen, since a lower level of lactose was present in the lactose-hydrolyzed and filtered milk compared to the conventional milk.
during heating. In conclusion, acetic and formic acid are formed during heat treatment and increase during storage. Acetic acid is considered to be the major degradation product of Amadori products and is a marker for the 1-deoxyosone pathway mainly via hydrolytic β-dicarbonyl cleavage, and formate is also formed from dicarbonyls (Hodge 1953; Brands and van Boekel 2001; Davidek et al. 2006; Smuda and Glomb 2013). The same trend in acid formation observed in all the milk types indicates similar deoxyosone degradation. This is intriguing because a higher amount of Amadori products in the lactose-hydrolyzed milk, as was indicated by a higher level of furosine, would imply that the level of deoxyosones formation is higher, which would lead to a higher level of acid formation in the lactose-hydrolyzed milk which was not observed. Consequently, it could be considered whether the acid formation is independent of the concentration and type of carbohydrates present in the milk during storage.

2-methylbutanal was one of the compounds analyzed by DHS-GC-MS in SIM mode (paper 2). The concentration of 2-methylbutanal in the conventional and lactose-hydrolyzed UHT milk during storage is presented in figure 12. Similar formation pattern was observed for 2-methylbutanal as for furosine (figure 11), thus, a lower initial 2-methylbutanal concentration was observed in the lactose-hydrolyzed milk, compared to the conventional milk. The concentration of 2-methylbutanal increased to a much greater extent in lactose-hydrolyzed milk compared to the constant level observed in the conventional milk during storage. 2-Methylbutanal is a Strecker aldehyde and is formed from the reaction between a dicarbonyl, such as 1-deoxyosone, and the amino acid isoleucine (Balagiannis et al. 2009). The result can be ascribed to higher reactivity of glucose and galactose compared to lactose (Naranjo et al. 2013). This is considered to be an important result for understanding the specific chemical reactions (e.g. Maillard reaction) occurring in lactose-hydrolyzed UHT milk during heating and storage.
Figure 12. Concentration of 2-methylbutanal in conventional and lactose-hydrolyzed UHT milk during storage.

2-methylfuran and 2-ethylfuran were observed to increase during storage for all the milk types. However, the increase was significantly higher in the conventional milk indicating that the formation of these furans were favored by lactose and not glucose or galactose (paper 2:figure 6). 2-Methylfuran and 2-ethylfuran can be formed from the Strecker degradation products of alanine (acetaldehyde) and threonine (lactaldehyde) as well as the presence of phenylalanine and degradation of carbohydrates in the milk (Limacher et al. 2008). In addition, the significant higher formation of furans from lactose, and not glucose or galactose, is supported by (Owczarek-Fendor et al. 2012). Thus, the hydrolysation of lactose seems to decrease the furan formation in the lactose-hydrolyzed milk during storage.

5.2.3 Final stage Maillard reaction

No chemical compounds associated with the final steps in the Maillard reaction were included in this study. However, the sensory descriptive analysis in paper 4 revealed differences in the attribute color saturation (saturation of white color) which is shown in figure 13. The conventional UHT milk had a higher degree of color saturation than the indirect lactose-hydrolyzed UHT milk at 3 months of storage and a significantly higher level was observed in the fresh and 3 months stored conventional milk compared with the direct lactose-hydrolyzed UHT milk at the same storage period. No significant difference in color saturation was observed between the milk types at 4 months of storage. This result indicates that the color saturation was only important the first three months of storage. However, during the DHS-GC-MS analyses clear visual differences between the conventional, filtered and lactose-hydrolyzed UHT milk were observed. The
lactose-hydrolyzed milk could easily be separated from the other milk types by the color; the lactose-hydrolyzed milk had a more yellow-brownish color in an early stage of storage, while the conventional and filtered milk kept the white color throughout the storage period. These visual differences observed indicate that the color saturation may be important after 4 months of storage. Figure 14 reveal that the color saturation increases throughout the storage time in the lactose-hydrolyzed milk and an increase is seen in the indirect lactose-hydrolyzed milk after 3 months of storage, while a constant level is observed in the conventional UHT milk. This pattern indicate that after 4 months of storage the color saturation in lactose-hydrolyzed milk could be higher than in the conventional milk if the increase continue during storage supporting the visual differences during DHS-GC-MS analysis. An increased color formation in the lactose-hydrolyzed milk indicates a higher protein polymerization for glucose and galactose and a more advanced Maillard reaction and formation of high molecular weight compounds, so called melanoidins, compared to lactose in the conventional milk (Brands et al. 2002). Thus, it could have been interesting to correlate the formation of color and Strecker aldehydes, and specifically 2-methylbutanal. The increased browning in lactose-hydrolyzed milk is supported by (Hayward 1977; Bunn and Higgins 1981; Kato et al. 1986; Kato et al. 1988; Oliver et al. 2006), Kato et al (1988) found an increased browning in ovalbumin-galactose>ovalbumin-glucose>ovalbumin-lactose model system.

![Figure 13. Color saturation in conventional indirect heated UHT milk (CONVI), lactose-hydrolyzed direct heated UHT milk (LHD) and lactose-hydrolyzed indirect heated UHT milk analyzed with sensory descriptive analysis.](image-url)
Chapter 6. Proteolytic activity

In this chapter the proteolytic activity will be discussed including the results obtained in the present project in relation to free amino terminals, free amino acids and proteolysis in the milk. Reverse phase high pressure liquid chromatography-mass spectrometry (RP HPLC MS) was used to analyze the protein profile, fluorescamine assay was used to analyze free amino terminals, and $^1$H NMR was used to analyze free amino acids in the milk, which was described in chapter 4.

6.1 Proteolytic activity

Modifications on proteins due to proteolytic activity have evoked a lot of attention, especially the peptides generated after the hydrolysis contributing to flavor and texture changes which is desired in cheese production but unwanted in milk (Nielsen 2002; Rauh et al. 2014). Native proteases such as the serine proteinase plasmin, the cysteine protease cathepsin D, and proteases produced by psychrotrophic bacteria are present in milk. Cathepsin D has shown to be increased in milk with increasing somatic cell count in the milk (Larsen et al. 2004). The psychrotrophs produce extracellular enzymes that are highly heat stable and may survive severe heat treatment such as UHT, plasmin may also survive heat treatments such as UHT heating while cathepsin D is heat unstable (Hurley et al. 2000; Hayes et al. 2001). The specificity of the proteases varies, but are preferentially caseins (Nielsen 2002; Wedholm et al. 2008). The peptides formed after hydrolysis may be analyzed and related to the specificities of the different proteases. One of the aims in the present study was to elucidate the effect of protein hydrolysis in the lactose-hydrolyzed UHT milk during storage and to obtain information about which proteins that were most affected by the proteolysis.

6.2 Proteolytic activity in conventional, filtered and lactose-hydrolyzed UHT milk

Free amino terminals (N-terminals) were analyzed, and quantified as leucine equivalents, with a fluorescamine assay, as discussed in paper 2. The level of free N-terminals increased in lactose-hydrolyzed milk during storage while in the conventional milk the level of N-terminals remained more or less constant.
throughout the storage period (paper 2:figure 10). This result indicates an increased proteolytic activity in lactose-hydrolyzed milk.

The protein profiles revealed that proteolytic activity was observed in all milk types for all intact proteins identified (β-CN, αs1-CN, αs2-CN, κ-CN and β-LG) during storage, which was presented in paper 3:table 2. Two-dimensional gel electrophoresis was also conducted on the milk proteins in the present study, which confirmed a proteolytic activity as well as glycation of the proteins in the milk (results not shown). The reduction of total intact CN during storage (sum of β-CN, αs1-CN, αs2-CN, κ-CN) was significantly higher in lactose-hydrolyzed milk with a 56-61% reduction compared to a 15% reduction in conventional and filtered milk. The difference between the lactose-hydrolyzed, filtered and conventional UHT milk became pronounced after 90 days of storage, where the reduction of total intact CN was observed in the lactose-hydrolyzed milk. The proteins that contributed most to the reduction in the lactose-hydrolyzed milk included β-CN and αs1-CN. Figure 14 presents the reduction in total intact CN, intact β-CN and αs1-CN during storage.

Free amino acids (alanine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine and valine) were analyzed with 1H NMR spectroscopy and were discussed in paper 2 and 3. With an exception for methionine, increased level of all identified free amino acids was observed in the lactose-hydrolyzed milk, while no increase (except for lysine) was observed in the conventional milk. These results are in good agreement with the results obtained by the fluorescamine assay and the protein profiles. Lysine increased in all milk types (paper 3:table 2), which indicates that the heat treatment applied to the milk did not inactivate all natural present indigenous enzymes. The lysine increase is most probably due to plasmin, which has high specificity for lysine, in addition, the cathepsin D was most probably inactivated during the heat treatment. Phenylalanine, tryptophan and tyrosine could not be identified in the conventional milk, which could be ascribed to that the indigenous enzymes primary produces large and small peptide fragments and not free amino acids (Sousa et al. 2001). The increase in free amino acids in the lactose-hydrolyzed milk indicates that the side-activity from the enzyme preparation applied to this milk is ascribed to unspecific enzymes that cleave one amino acid at a time e.g. amino peptidases (Gonzales and Robert-Baudouy 1996).

The concomitant higher level of free amino terminals, free amino acids and the reduction in the level of caseins in lactose-hydrolyzed milk compared to the conventional and filtered milk strongly indicates that this is due to proteolytic
activity from the β-galactosidase enzyme preparation added to the lactose-hydrolyzed milk. The filtered milk was included in the study to elucidate what effect filtration had on the milk and milk proteins. However, the comparable behavior of the filtered and conventional milk revealed that the milk was not affected by this processing step. This is an interesting and important result that must be investigated further in relation to the type of responsible enzymes as well as the type of peptides that are generated in the milk during storage. Furthermore, it is worth noticing that the level of amino acids increased throughout the storage time in the lactose-hydrolyzed milk, while the reduction in total intact casein was first observed after 90 days. This indicates that the protein hydrolysis must reach a specific level to be detected by the RP-HPLC MS. In addition, the amino acid increase may reflect the total hydrolysis of all the proteins present in the milk.

The increased proteolytic activity observed in the lactose-hydrolyzed UHT milk is supported by (Tossavainen and Kallioinen 2007). Tossavainen and Kallioinen (2007) analyzed UHT milk stored for 4 months at 22 °C by SDS-page and observed an increased proteolytic activity in the lactose-hydrolyzed milk during storage compared to the conventional milk. The most hydrolyzed proteins were αs1-CN, αs2-CN and β-CN and the conclusion from the study was that the enzyme applied to the milk to hydrolyze lactose contained proteolytic side activities. Similar results were obtained in another study on extended shelf-life heated lactose-hydrolyzed milk which revealed an increased proteolytic activity in the lactose-hydrolyzed milk compared to the conventional milk after two weeks of storage (Kallioinen and Tossavainen 2009).
Figure 14. Relative quantification of total-CN, αs1-CN and β-CN in conventional, lactose-hydrolyzed and filtered UHT heated milk during storage at 22 °C.
Chapter 7. Lipid oxidation and thermal degradation

In this chapter lipid oxidation will be discussed including the results obtained in the present project in the concentration of aldehydes, ketones and furans in the milk. DHS-GC-MS was used to analyze the volatile compounds present in the milk, as described in chapter 4.1.1.

7.1 Introduction to lipid oxidation

Lipid oxidation is, in addition to the Maillard reaction, one of the most important sequences of reactions taking place in food products. Oxidation of lipids, primary the unsaturated fatty acids is catalyzed by heat (Vazquez-Landaverde et al. 2005; Costa et al. 2011), light (Barriuso et al. 2012), enzymes, transition metals and by microorganisms (Walstra et al. 1999; Shahidi and Zhong 2010). The oxidation of the fatty acids generates primary oxidation products including lipid hydroperoxides (LOOH). These peroxides are unstable and decompose to radicals which react with new lipid molecules and initiate a propagation process in the so called autoxidation. This process will proceed until the radicals potentially react with antioxidants or proteins which may terminate the process (Frankel 1982; Alaiz et al. 1997; Yilmaz and Toledo 2005; Elias et al. 2008; Shahidi and Zhong 2010). The hydroperoxides can also be decomposed to secondary oxidation products such as aldehydes and ketones.

Psychrotrophic bacteria are favored by cold storage and may contribute to lipid degradation by the formation of the heat stable extracellular enzymes such as lipases and proteases. This could be relevant in the milk prior heat treatment when the milk is stored cold. The bacteria produce these enzymes in the late log phase and early stationary phase, thus a rather high concentration of bacteria is needed to produce sufficient levels of enzymes that can degrade the lipids in milk. However, for UHT treated milk, during the long-term storage at higher temperatures the bacterial lipases may contribute to the degradation of lipids (Czerniewicz et al. 2006; Gargouri et al. 2013).

7.2 Effect of lactose-hydrolysis on lipid oxidation

In paper 1 it was revealed that the ketone concentration varied between the milk types (conventional, filtered and lactose-hydrolyzed UHT milk) as well as within the batches of each milk type. In addition, the conventional UHT milk
had overall a higher concentration of all ketones identified in the milk during storage compared to the lactose-hydrolyzed UHT milk which was presented in paper 2:figure 4. A similar formation rate was observed in all milk types during storage, although the formation rate of 2-pentanone, 2-heptanone and 2-nonanone were significantly higher in the lactose-hydrolyzed milk compared to the conventional milk, which was also the most abundant compounds in all milk types (paper 2:figure 4). Ketones are formed from oxidation of β-ketoacids and are highly temperature-dependent. Consequently, similar levels of ketones could have been expected for the indirect heated conventional and lactose-hydrolyzed milk, while a lower level of ketones may have been expected in the direct lactose-hydrolyzed milk compared to the indirect heated milk. However, no difference was observed between the direct and indirect heated lactose-hydrolyzed milk, indicating that the different heat-load did not affect the formation of ketones. A possible explanation for the variation within the batches could be differences in holding times before the heat treatment, as well as different raw milk quality which contributes to variations in bacterial growth and bacterial production of enzymes (Gargouri et al. 2013). These enzymes may affect the stability of the lipids prior to the heat treatment and contribute to a higher level of lipid degradation during the heat treatment. The higher level of ketones in the conventional UHT milk compared to the lactose-hydrolyzed UHT milk, and the similar ketone levels in the lactose-hydrolyzed milk types indicate that the hydrolysis of lactose affect the ketone formation more than the heat load.

No significant changes in the level of lipid oxidation derived aldehydes were observed in any of the milk types during the 9-months storage period (paper 2 and 3). Similar levels were found for the aldehydes in the lactose-hydrolyzed and conventional UHT milk during storage, probably due to the same concentration of fat in all milk types. From these results it were concluded that the lactose hydrolysis did not affect the lipid oxidation derived aldehydes.

Vazquez-Landaverde et al. (2005) investigated volatile compounds in raw, pasteurized and UHT heated skim and whole milk by SPME (Vazquez-Landaverde et al. 2005). The study revealed that the level of ketones increased with increasing heat load and that 2-heptanone and 2-nonanone were two of the most abundant ketones in all the milk supporting the results observed in paper 2. In addition, the study showed that the aldehydes (hexanal, furfural, heptanal, octanal, nonanal and decanal) increased with increasing heat treatment and increasing lipid content, although to a lower degree compared to the ketones. Contarini et al. (1997) investigated direct heated UHT milk stored for 3 months
at room temperature using dynamic headspace GC-MS and observed an increased concentration in the identified ketones 2-butanone, 2-pentanone and 2-heptanone during storage. In addition, the aldehydes identified (pentanal, hexanal and heptanal) in the skim milk increased during storage, while they decreased in the whole milk (Contarini et al. 1997). Valero et al. (2001) found an increased level of hexanal in whole milk and a decrease in skim milk, including high variation of the compounds during the storage time. These contradictory results indicate that the method applied to analyze aldehydes is important for the content of aldehydes, that the aldehydes are dependent on storage conditions as well as concentration of lipids.

In paper 2, 2-pentylfuran was observed to increase significantly in conventional UHT milk compared to the lactose-hydrolyzed UHT milk during storage. 2-Pentylfuran is found to be derived from linoleic acid (Frankel 1982; Min and Boff 2002; Perez Locas and Yaylayan 2004; Becalski and Seaman 2005; Hasnip et al. 2006; Zhang et al. 2012), and the lower formation rate in lactose-hydrolyzed milk could indicate that an antioxidant effect from the more progressed Maillard reaction and from the proteolytic activity forming antioxidative compounds may decrease the formation of lipid oxidation (Morales and Jiménez-pérez 2001; Clausen et al. 2009; Gu et al. 2009).
Chapter 7. Lipid oxidation and thermal degradation
Chapter 8. Sensory characteristics

In this chapter the sensory characteristics of milk will be discussed including results obtained in the present project. It will be focused on results presented in paper 4 which related the chemical compounds, in the form of volatile compounds, carbohydrates and primary amines with sensory characteristics. Sensory descriptive analysis was used to describe the sensory characteristics of milk and other analyzing techniques applied were DHS-GC-MS, 1H NMR spectroscopy and fluorescamine assay (described in chapter 4).

8.2 Sensory and chemical analysis of milk

8.2.1 Aroma and flavor of milk

Aroma and flavor are both very important sensory attributes of milk products (Thomas 1981; Giusti et al. 2007; Kokkinidou and Peterson 2014) and if the aroma and/or flavor are altered due to bacterial, enzymatic or chemical changes the consumer acceptance will be affected. The aroma and flavor of milk are determined by the content of aroma active volatiles at different concentrations. Many compounds are found in concentrations below the threshold values but still contribute to the distinct impression of milk due to mixture enhancement (Badings et al. 1981; Carunchia and Drake 2007). In lactose-hydrolyzed milk, it is a challenge to maintain sensory characteristics identical to conventional milk (Adhikari et al. 2010), since the change in carbohydrates may alter the perceived sweetness and also affect other sensory characteristics through mixture enhancement.

The aroma and flavor of raw milk is refined and balanced, generated from a complex mixture of volatile compounds present at different concentrations. Dimethyl sulfide, diacetyl, 2-methyl-1-butanol and different aldehydes are important for the aroma and flavor of raw milk (Belitz et al. 2001; Al-Attabi 2009). When milk is heat-treated the aroma and flavor is changed markedly. Heat-treated milk is described to have a cooked, heated, stale and flat flavor (Badings et al. 1981; Zabbia et al. 2012). The aroma and flavor is mainly determined by sulfide compounds, ketones, aldehydes and lactones, formed from thermal degradation of proteins, lipid oxidation and the Maillard reaction. However, the aroma and flavor depends on the heat load applied as well as level of dissolved oxygen (Thomas et al. 1975; Contarini et al. 1997; Walstra et al. 1999; Vazquez-Landaverde et al. 2005). The aroma and flavor of pasteurized
milk is highly accepted among people in Scandinavia, as well as in the US, while the flavor of UHT milk is less accepted in Scandinavia and US but still popular in other European countries (Clare et al. 2005; Kokkinidou and Peterson 2014).

As found in the project, the aroma and flavor of milk constantly change during storage due to the different chemical reactions taking place in the milk. These chemical changes were discussed in chapter 4, 5 and 6, and paper 2, 3, and 4, and an overview can be seen in figure 15.

8.2.2 Sensory descriptive analysis and chemical analysis of conventional and lactose-hydrolyzed UHT milk during storage

In the present study, boiled milk flavor (figure 15) was present in a higher level in conventional UHT milk compared with the two lactose-hydrolyzed milk types after 3 months of storage (paper 4:figure 1). In addition, boiled milk aroma increased from fresh (0 months) to 3 months of storage in conventional and indirect heated lactose-hydrolyzed UHT milk (figure 15). This indicates that the different heat processes, direct and indirect UHT, did not affect the boiled milk flavor but had an effect on the boiled milk aroma. In addition, the hydrolyzation did have an effect on the boiled flavor intensity. Glycated β-LG has a higher heat stability compared to unglycated β-LG which might explain the observed effect in boiled flavor of the lactose hydrolysis in the milk (Liu and Zhong 2013). Tossavainen (2008) used a trained sensory panel to investigate lactose-hydrolyzed and conventional UHT milk. The sensory analysis revealed similar development of cooked (boiled), heated and burned flavor in both milk types suggesting that cooked flavor is mainly dependent on the protein denaturation and not the type of carbohydrates in the milk (Tossavainen 2008). This result was inconsistent with the results in the present study. Cooked flavor is directly linked to the heat processing as a result of the thermal denaturation of the serum proteins, which expose thiol and sulfide groups that contribute to the formation of sulfur containing volatiles (Hutton and Patton 1952). A reduced protein denaturation and a concomitant reduction in cooked flavor has been demonstrated in milk subjected to direct UHT processing compared with indirect UHT processing (Al-Attabi 2009). However, this was not observed for the lactose-hydrolyzed UHT milk in paper 2, 3 and 4.

DMDS and DMTS were the only sulfides identified in the study (paper 2, 3 and 4). DMDS remained constant and below its threshold (7.6 µg/liter water (Belitz et al. 2001)) value in all milk types throughout the study (paper 3:table 2).
DMTS increased in the lactose-hydrolyzed milk during the first 50 days of storage followed by a decline, while in the conventional milk DMTS decreased steadily throughout storage (paper 2:figure 7). After 3 months of storage the conventional UHT milk had a lower level of DMTS compared to the direct lactose-hydrolyzed UHT milk (paper 4). The decline in DMTS is probably due to oxidation, or conversion to other sulfide containing compounds (Rerkrai et al. 1987; Al-Attabi 2009). The sulfides may have contributed to the boiled milk aroma in the first period of storage.

Rerkrai et al. (1987) studied sensory characteristics of direct heat-treated UHT milk for 24 weeks of storage and found that the cooked flavor decreased while the stale flavor increased throughout storage (Rerkrai et al. 1987). In this study stale flavor was evaluated to be more intense in the 3 months stored direct lactose-hydrolyzed UHT milk compared to the 3 months stored conventional UHT milk shown in figure 15 (paper 4). The off-flavor observed in the lactose-hydrolyzed milk was expected to be related to stale flavor formed in the milk during storage. Therefore, a correlation between methyl ketones, aldehydes and stale flavor was investigated by partial least square (PLS) regression presented in paper 4:figure 2. The expected off-flavor could not be entirely related to the stale flavor formed during storage as the stale flavor intensity was observed to be highest in fresh conventional UHT milk compared to all the other milk types at every analyzing time. The PLS regression revealed that 2-butanone, 2-pentanone, 2-heptanone, 2-nonanone, heptanal, octanal and nonanal were found to be most important for the stale flavor (paper 4:figure 2). As discussed in chapter 7 and paper 2 the ketones increased during storage in all milk types which was also shown in paper 4. In addition, no pronounced difference in ketone formation during storage was observed between the conventional and lactose-hydrolyzed milk (paper 2:figure 4). From this result it was concluded that the ketones do not have a major role in the formation of off-flavor in lactose-hydrolyzed UHT milk during storage.

The lipid oxidation derived aldehydes identified and presented in paper 2 and 3 did not increase during storage. However, in the results presented in paper 4 the conventional milk had a higher level of hexanal compared to the lactose-hydrolyzed milk after three months of storage and octanal and decanal significantly increased during 4 months of storage in all milk types. Based on these findings, lipid oxidation derived aldehydes are not expected to contribute greatly to the off-flavor in lactose-hydrolyzed milk.
Furthermore, Valero et al. (2001) studied the sensory properties of conventional UHT milk and found that a stale flavor appeared after three months of storage. Increased aldehyde concentration during storage has been observed to correlate with stale flavor, as well as Strecker degradation products (Jeon et al. 1978; Ledl and Schleicher 1990; Perkins and Elliott 2005; Zabbia et al. 2012). Vazquez-Landaverde (2005) compared the concentration of aldehydes in UHT, pasteurized and raw milk. In general, nonanal and decanal were found to contribute to raw milk flavor and for UHT flavor, octanal, hexanal, 2-methylbutanal, 3-methylbutanal and 2-methylpropanal were found to be important, even though they were not as abundant as the ketones in the UHT milk.

In paper 2, 2-methylbutanal was identified and quantified in lactose-hydrolyzed and conventional UHT milk. The threshold value of 2-methylbutanal is 4 µg/liter water (Belitz et al. 2001) and in the lactose-hydrolyzed milk 2-methylbutanal was quantified to 7-8 µg/kg after 9 months of storage while the concentration remained constant below 1 µg/kg in the conventional UHT milk throughout the storage period (paper 2:figure 5). Intriguingly, the concentration of 2-methylbutanal in the lactose-hydrolyzed milk reached the threshold value when the milk was 4.5 months old, which is close to the shelf-life of this milk type. The high concentration of 2-methylbutanal observed in the lactose-hydrolyzed milk during storage would definitely contribute to the flavor in the milk.

Even though no decrease in carbohydrate concentrations were observed from the 1H NMR spectroscopy as discussed in chapter 5 and in paper 3:table 2 the sweet taste decreased in all milk types during storage (figure 15) (paper 4). This finding can possibly be described by a reduction of available carbohydrates due to the Maillard reaction (Chávez-Servín et al. 2006) and possibly by the masking effect from formation of bitter tasting compounds during storage (Beck et al. 2014). In paper 4, an increased bitter taste was observed in the lactose-hydrolyzed milk compared to the conventional milk. In addition, the bitter taste intensity was correlated with the level of free amino terminals which was higher in the lactose-hydrolyzed milk compared to the conventional milk (figure 16) (paper 2 and 4). The increased level of free amino terminals in the lactose-hydrolyzed milk support the results in paper 3 which revealed a reduction in total intact CN in the lactose-hydrolyzed milk after 90 days of storage, while the reduction was less pronounced in the conventional and filtered UHT milk (figure 14). The reduction in total intact CN was primary due to the proteolysis of β-CN and αs1-CN. This result is supported by McKellar (1981) who
demonstrated a correlation between levels of proteolysis and formation of bitter flavor in UHT milk (McKellar 1981). The bitter flavor may be due to increased level of hydrophobic bitter peptides and amino acids due to casein hydrolysis (Guigoz and Solms 1976; Ney 1979; Harwalkar et al. 1993; Gomez et al. 1997; Kilara and Panyam 2003).

Figure 15. Intensity of sensory attributes during 4 months of storage in conventional UHT milk (CONVI), lactose-hydrolyzed direct heated UHT milk (LHD) and lactose-hydrolyzed indirect heated UHT milk (LHI). Lower case letters indicate statistical difference between different milk at the same storage time (p<0.05).
Figure 16. Correlation between the level of primary amino groups (X-axis) and bitter taste intensity of three different samples of milk (direct and indirect heated lactose-hydrolyzed UHT milk and indirect heated conventional UHT milk) stored for four months.
Chapter 9. Effect of heat treatment and storage on lactose-hydrolyzed milk

In this chapter the relation between the different compounds identified in the present study will be discussed. The Maillard reaction, proteolysis and lipid oxidation are all parameters that have a great influence on the chemical changes in the milk. This chapter aims to connect the reactions and the most important compounds identified into a coherent perspective. In figure 17 a cascade of reactions mainly based on the Maillard reaction compounds identified in this study, however, additional side activities such as the carbohydrate degradation, formation of amino acids by proteolysis, and thermal degradation of lipids are included in the cascade.
Figure 17. Overview of the compounds identified in the conventional, filtered and lactose-hydrolyzed milk. The compounds were identified with GC-MS (blue rectangles), NMR (orange rectangles), HPLC (green rectangles) and fluorescamine assay (pink rectangle). A dashed outline indicates increase of compounds in lactose-hydrolyzed UHT milk, a dotted outline indicates increase of compounds in conventional UHT milk, a solid outline indicates no difference between lactose-hydrolyzed and conventional UHT milk during storage.
The Maillard reaction is highly dependent on the concentration of carbohydrates and proteins available in the milk during heat treatment and storage. As was observed in the formation of the volatile compounds furosine, 2-methylbutanal and formate, shown in figure 12, 13 and paper 3:table 2, respectively, the carbohydrate concentration during heat treatment had a major effect on the initial level of the formation of the compounds. In addition, during storage the carbohydrate type affected the formation rate tremendously in furosine and 2-methylbutanal.

The free amino acid isoleucine formed in the milk due to proteolysis had a major effect on the formation of 2-methylbutanal and in figure 18 it can be seen that 2-methylbutanal and isoleucine showed similar development pattern. Without the involvement of amino acids in the Maillard reaction, less advanced Maillard reaction products would be formed, as demonstrated in figure 18 by the constant low concentration of 2-methylbutanal in the conventional UHT milk during storage (paper 2:figure 13). Based on this fact it is proposed that the proteolysis present in the lactose-hydrolyzed milk gives rise to free amino acids that can be included in the Maillard reaction cascade, such as Strecker degradation and give rise to flavor-contributing compounds such as 2-methylbutanal.
Chapter 9. Effect of heat treatment and storage on lactose-hydrolyzed milk

Figure 18. GC-MS analysis of 2-methylbutanal and 1H NMR analysis of isoleucine in conventional and lactose-hydrolyzed UHT milk during storage.

The importance of amino acids in the Maillard reaction has been highlighted in earlier studies and also reviewed (Izzo and Ho 1992; van Boekel 2006; Balagiannis et al. 2009). Balagiannis et al. (2009) investigated the importance of the level of glucose in the formation of Strecker aldehydes, and observed a clear correlation between the level of glucose and the formation of Strecker aldehydes. However, the study also revealed that the levels of the individual amino acids included in the formation of the specific aldehyde were highly important. The amino acids should have more focus in relation to the formation of flavor compounds in milk and especially lactose-hydrolyzed milk. However, Izzo and Ho (1992) as well as van Boekel (2006) have discussed the importance of the arrangement of amino acids in peptides and proteins, which also may contribute to the formation of flavor compounds. Martins and van Boekel (2005) investigated the importance of the initial concentration of glucose and glycine for the formation of organic acids, glucosones and melanoidins formed in the Maillard reaction (Martins and van Boekel 2005). No significant difference was found for the reaction rate when the initial concentration of the carbohydrates
was two times higher than the proteins concentration or when the protein concentration was two times higher than the carbohydrate concentration. This indicates that the precursor with lowest concentration limits the formation of the organic acids, glucosones and melanoidins in the Maillard reaction. As already discussed in this chapter, a lower initial concentration was observed in many of the identified compounds in the milk as presented in paper 2 and 3, and the 40% lower level of lactose during heat treatment was important for the protein and carbohydrate condensation. Considering the lactose-hydrolyzed UHT milk, and the higher level of Maillard reactions taking place during storage, it is expected that the fact that the milk is heated with a lower lactose concentration has a positive influence. This may decrease the reaction rate of the formation of Maillard reaction compounds in the initial storage period, and should decrease the formation of products in the advanced and final stage of the Maillard reaction compared to as if the lactose had been hydrolyzed before the heat treatment.

In the present study, comparing conventional and lactose-hydrolyzed UHT milk, lipid oxidations are not the major reaction contributing to off-flavor in lactose-hydrolyzed milk during storage. However, even though the lipid oxidation does not directly contribute to flavor in the lactose-hydrolyzed milk the lipid oxidation products may be included in reactions with carbohydrates, proteins as well as in the Maillard reaction at different stages (Whitfield and Mottram 1992). Yaylayan (1997) states that all the precursors in milk such as carbohydrates, amino acids and lipids are divided into pools that can react and degrade to reactive compounds individually but also across the pools (Yaylayan 1997). This contributes to a highly complex cascade of reactions.

Zamora and Hidalgo (2005) reviewed the reaction products formed in the Maillard reaction and lipid oxidation and discussed how the two reactions interacted and concluded that the Maillard reaction may both increase lipid oxidation by phospholipid glycation and reduce the lipid oxidation by formation of antioxidant compounds, and the lipid oxidation may also increase the formation of Maillard reaction compounds (Zamora and Hidalgo 2005). The Maillard reaction can in the advanced and final stage generate compounds, such as melanoidins, that have an antioxidant activity which thereby can reduce the lipid oxidation (Alaiz et al. 1999). The lipids are included in the Maillard reaction by the amino group of the phospholipid and by the aldehydes and ketones formed from the fatty acid oxidation (Zamora and Hidalgo 2005). In addition, the unsaturated aldehydes may react with hydrogen sulfide and thereby inhibit the formation of further reactions to sulfide containing compounds that
contribute to cooked flavor of the product. The lipid oxidation may also generate dicarbonyls such as glyoxal and methylglyoxal, as well as radicals, highly reactive with proteins and Maillard reaction compounds.

An extensive Maillard reaction may decrease the quality of milk considerably. However, the prevalence of proteolytic enzymes that degrade proteins into amino acids, included in the Maillard reaction, and bitter tasting peptides may be just as important for the milk quality during storage than the Maillard reaction itself. Hence, the enzymatic hydrolysis of lactose into glucose and galactose contributes to an increased Maillard reaction due to the increased reactivity of the monosaccharides. However, side-activities from the enzyme preparation may contribute more to the increased reactions observed in the lactose-hydrolyzed milk through the formation of amino acids and bitter peptides.
Chapter 10. Conclusion and future perspectives

10.1 Conclusion

The overall aim of the present PhD study was to elucidate differences between conventional and lactose-hydrolyzed UHT milk in relation to formation of off-flavor during a nine-month storage period.

Specific Maillard reaction compounds were identified in the conventional and lactose-hydrolyzed UHT milk by DHS-GC-MS, NMR and RP HPLC MS. The study revealed that the Amadori-product indicator, furosine, and the Strecker degradation compound 2-methylbutanal increased in the lactose-hydrolyzed UHT milk during storage, while the concentration remained constant in the conventional UHT milk (paper 2). This finding indicates that the hydrolysis of lactose to the more reactive carbohydrates glucose and galactose appears to have a major effect on the formation of Maillard reaction compounds. The study also revealed that the initial concentration of furosine, formate and specific volatile compounds e.g. 2-methylbutanal were higher in the conventional UHT milk than in lactose-hydrolyzed and filtered UHT milk. This likely reflects that the concentration of lactose affects the degree of formation of these compounds during the heat treatment.

Chromatographic profiling of the intact proteins in the milk revealed that hydrolysis was present during storage in both lactose-hydrolyzed UHT and conventional UHT milk, although more apparent in the lactose-hydrolyzed milk compared to the conventional milk (paper 3). Filtered UHT milk was also included in the study to follow the processing of the lactose-hydrolyzed milk, and a similar protein hydrolysis in the filtered and conventional milk was observed. Consequently, it is concluded that the β-galactosidase enzyme preparation contains side-activities that causes protein hydrolysis in the lactose-hydrolyzed UHT milk.

An interesting and concomitant pattern was observed in the formation of 2-methylbutanal and the hydrolysis of β- and αs1-CN in the lactose-hydrolyzed UHT milk. After 90 days of storage the level of 2-methylbutanal was higher in the lactose-hydrolyzed UHT milk than in the conventional milk, and concomitantly a pronounced decrease in the level of intact β- and αs1-CN was initiated. Intriguingly, the shelf-life of lactose-hydrolyzed UHT milk is also approximately 90 days. Thus, a connection seems to be plausible, which implies
that these chemical changes in the lactose-hydrolyzed milk may be crucial for the shelf-life.

Sensory analysis revealed that stale flavor increased in both lactose-hydrolyzed UHT and conventional UHT milk during 4 months of storage. GC-MS analysis revealed that the stale flavor was related to an increased level of ketones and specific aldehydes: 2-methylbutanal, octanal and decanal (paper 4). The relation between stale flavor and 2-methylbutanal could indicate that 2-methylbutanal is a decisive compound for the formation of off-flavor observed in the lactose-hydrolyzed milk since a higher concentration was observed in this milk type compared with the conventional milk during storage.

Sensory analysis also revealed a higher bitter taste score in the lactose-hydrolyzed milk than in the conventional UHT milk (paper 4). Intriguingly, the bitter taste was correlated with the level of free amino terminals in the milk analyzed by fluorescamine assay, which was higher in the lactose-hydrolyzed milk compared to the conventional milk. The correlation between bitter taste and increased level of free amino terminals is an important result in relation to understand the consequence of an increased protein hydrolysis in the lactose-hydrolyzed milk. The bitter taste and the higher level of free amino terminals in lactose-hydrolyzed milk could most probably be related to the more extensive hydrolysis of the proteins β- and αs1-CN (paper 3).

When comparing two lactose-hydrolyzed milk types exposed to direct and indirect UHT treatment, respectively, no differences in the formation of Maillard reaction compounds or protein hydrolysis were observed (paper 2 and 3). Consequently, it can be concluded that the type of sugar and protein hydrolysis most probably affect the chemical reactions taking place in the milk to such an extent that the effect of the difference in the heat load becomes diminishing.

Batch differences were observed within each milk type, which indicates that it is difficult to produce commercial milk without variation (paper 1). The variation was mostly apparent in the concentration of ketones, indicating that the variation is dependent on the lipids in the milk. Batch variation can probably be reduced by making the time before heat treatment of the milk as short as possible to avoid degradation of lipids by naturally present lipases or psychrotrophic produced enzymes. The variation present between different batches may not be noticeable or important for the consumers. However, when analytically investigating the milk the variation had considerable impact on the results.
The Maillard reaction is a highly complex reaction including many different reaction pathways. This study has contributed to an increased understanding of how Maillard reaction products are generated in milk during storage. In addition, the study has revealed how protein hydrolysis affects Maillard reaction product formation, and how the proteolysis may contribute to bitter taste in the milk. Further investigations are needed to find the relation between milk constituents and chemical changes that can be linked to the decreased shelf-life of lactose-hydrolyzed UHT milk.
10.2 Future perspective

For future improvements in the production of long-term stored lactose-hydrolyzed milk, the raw material should be considered as a key parameter to obtain a high-quality product and to eliminate variation between batch productions. It is recommended that the raw milk is of highest quality and that the transportation to and holding time at the dairy are as short and cold as possible to avoid unnecessary degradation of proteins and lipids in the milk. Based on the results of the present project it seems advantageous that the enzyme preparation applied to the milk to hydrolyze lactose is of highest purity, to avoid side-activities from other enzymes present in the preparation. Consequently, investigating the enzyme preparation in relation to purity and to the side activities that contribute to the degradation of the proteins in the milk could be highly relevant and beneficial for further advancements in the development of high-quality lactose-hydrolyzed milk products.

This study revealed that especially the hydrolysis of two caseins was enhanced in the lactose-hydrolyzed milk, and there was a strong indication that this hydrolysis could be correlated to the development of an increased bitterness. Therefore these casein degradation products should be investigated further in order to find the specific peptides that contribute to bitterness. The characterization of these peptides and the cleavage sites will also contribute to the identification of the enzymes responsible for the hydrolysis.

Future research in lactose-hydrolyzed milk could include a more specific analysis of the phenolic compounds in the milk. It is known that the β-galactosidase preparation may contain the enzyme arylsulfatase, which hydrolyze alkyl phenols substituted with a sulfate group that can give off-flavor. In addition, more Strecker degradation compounds should be analyzed and identified in order to correlate the free amino acids with the formation of Maillard reaction compounds. It could also be relevant to correlate the bacteria and enzyme activity in the milk prior to the heat treatment with the ketone concentration and fatty acid composition in the milk to obtain an increased understanding for how the raw milk affects the end product.

Another chemical reaction that may pose effect on the milk quality is glycation of proteins. The present study revealed a molecular weight shift of +162 Da and +324 Da for the intact proteins that indicated glycation. However, the result did not reveal which of the proteins that was glycated or the degree of glycation. The degree of glycation in the different milk types can be correlated to reactions taking place in the Maillard reaction during storage, and would therefore be
highly relevant to analyze. In addition, it could be interesting to elucidate which proteins in the milk that was most disposed for glycation and if a relation between degree of glycation and proteolysis could be observed.

For further research it could be very exciting to investigate if the milk genome could be used to find a type of milk that was suited for long-shelf life, highly heat-treated, milk. As milk genomic and metabolomic projects have been used to find specific milk types with high coagulating proteins for cheese production (Sundekilde et al. 2011), milk with less level of proteins prone for glycation and hydrolysis could be found and used specifically for UHT milk.

The β-galactosidase enzyme used to hydrolyze the lactose in the milk may contribute to a formation of oligosaccharides (Gänzle et al. 2008; Coulier et al. 2009; Gosling et al. 2011; Ruiz-Matute et al. 2012). Oligosaccharides have been shown to have prebiotic effects (Ben et al. 2008) and consequently, the oligosaccharides contribute to a better micro-flora in the intestine. It could be relevant to investigate the positive effect of adding β-galactosidase to milk by investigating the oligosaccharides formed in more detail by NMR spectroscopy.

It is desirable to decrease the Maillard reaction in milk during storage both from a product and a nutritive perspective. A previous study has shown that antioxidants may decrease the formation of Maillard reaction compounds in conventional UHT milk (Kokkinidou and Peterson 2014). Consequently, it would be interesting to investigate how different antioxidants added to milk affect the degree of Maillard reaction in lactose-hydrolyzed UHT milk during storage. In addition, a sensory descriptive analysis could be applied to the study to obtain information about the antioxidant effect on the sensory characteristics of the milk. Overall, there are many unexplored factors and possibilities to optimize lactose-hydrolyzed milk products and several issues to address in future research within this field, which can be expected to increase as a result of an increasing interest in lactose-reduced products from consumers world-wide.
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11. References


11. References


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11. References


11. References


Paper 1

Volatile component profiles of conventional and lactose-hydrolyzed UHT milk- a dynamic headspace gas chromatography-mass spectrometry study


Volatile component profiles of conventional and lactose-hydrolyzed UHT milk—a dynamic headspace gas chromatography-mass spectrometry study

Therese Jansson • Sidsel Jensen • Nina Eggers • Morten R. Clausen • Lotte B. Larsen • Colin Ray • Anja Sundgren • Henrik J. Andersen • Hanne Christine Bertram

Abstract Lactose-hydrolyzed milk gains still increasing market share, and understanding the chemical characteristics of lactose-hydrolyzed milk products is important for the dairy industry. The aim of the present study was to identify and compare volatile compounds of commercial lactose-hydrolyzed and conventional ultra-high temperature (UHT) milk. For this purpose, the volatile compounds of lactose-hydrolyzed (<1% lactose), conventional (100% lactose), and filtered (60% lactose) UHT-treated milk were extracted using dynamic headspace sampling and analyzed by gas chromatography-mass spectrometry (GC-MS). A total of 24 volatile compounds were identified including ketones, aldehydes, and sulfides. Overall, principal component analysis (PCA) showed grouping of the different milk types, with loadings indicating a higher concentration of ketones in conventional versus lactose-hydrolyzed UHT milk, but PCA also indicated a marked batch-to-batch variation. Elucidation of individual volatile compounds detected also revealed that the content of ketones in general was higher in conventional UHT milk than in lactose-hydrolyzed milk; however, no significant differences in the volatile compound profiles could be identified between the various milk types as a result of the batch-to-batch variation. The present study
highlights a useful analytical method based on dynamic headspace sampling and GC-MS to profile volatiles important for the flavor characteristics of lactose-hydrolyzed and conventional UHT milk. In addition, the present study reveals that a considerable batch-to-batch variation exists in industrially produced batches of lactose-hydrolyzed UHT milk, which must be considered an important challenge for the dairy industry.

Keywords  Lactose-hydrolyzed milk · UHT · Milk batch variations · Dynamic headspace sampling · GC-MS

Abbreviations

DHS Dynamic headspace sampling  
DMDS Dimethyl disulfide  
DMS Dimethyl sulfide  
DMSO Dimethyl sulfoxide  
DMTS Dimethyl trisulfide  
FWER Family-wise error rate  
GC Gas chromatograph  
IS Internal standard  
LME Linear mixed effect  
LOD Limit of detection  
LOQ Limit of quantification  
MR Maillard reaction  
MS Mass spectrometry  
nd Not detected  
nq Not quantified  
PC Principal component  
PCA Principal component analysis  
REML Restricted maximum likelihood  
TIC Total ion current  
UHT Ultra-high temperature  
UV Unit variance

1 Introduction

More than 70% of the world’s population is unable to digest lactose as a consequence of a reduced or nonexistent β-galactosidase activity (Messia et al. 2007; Sahi 1994). This is referred to as “lactose malabsorption” or “lactose intolerance” depending on the degree of reduction in β-galactosidase activity. These conditions give rise to bacterial fermentation of lactose in the colon, resulting in the production of copious amounts of gas (a mixture of hydrogen, carbon dioxide, and methane) that causes abdominal symptoms such as bloating, cramps, and nausea (Zhong et al. 2004). During the past decade, several lactose-hydrolyzed milk products have been introduced to the commercial market to meet the needs of consumers with lactose intolerance.

High-quality lactose-hydrolyzed milk may be produced using filtration to remove approximately 40% of the lactose. The remaining lactose is enzymatically hydrolyzed by the addition of β-galactosidase, producing the monosaccharides glucose and...
galactose. The decomposition of lactose to glucose and galactose in milk may influence the type and extent of chemical reactions in the milk taking place during processing and storage. For instance, Kato et al. (1986, 1988) reported that browning reactions and protein polymerization, both of which are characteristic for the advanced stages of the Maillard reaction (Brands et al. 2002), proceed more extensively in protein-galactose and protein-glucose models than in an equivalent protein-lactose model during heating (Brands et al. 2000). Consequently, lactose-hydrolyzed milk is expected to be more susceptible to Maillard reaction and Strecker degradation during storage than nonhydrolyzed ultra-high temperature (UHT) milk (Messia et al. 2007).

Various forms of thermal processing are used to prevent enzymatic and microbial spoilage and thereby extend the shelf life of milk. One commonly used heat treatment in the dairy industry is UHT processing (135–150 °C, 2–20 s; Kessler 2002). UHT-treated milk has a long shelf life and may be stored at room temperature for up to 8 months. However, it is well established that severe heat treatment and storage at ambient temperature lead to the development of off-flavors in milk (Jeon et al. 1978; Rerkrai et al. 1987; Valero et al. 2001).

Extensive investigations have been carried out to characterize the chemical changes giving rise to unwanted off-flavors in heat-treated milk (Rerkrai et al. 1987; Valero et al. 2001; Coppa et al. 2011; Vazquez-Landaverde et al. 2005; Tosó et al. 2002; Contarini and Povolo 2002; Contarini et al. 1997). The severity of the heat treatment strongly affects the resulting volatile profile of milk. In particular, UHT milk is characterized by an increase in the amount of ketones (Vazquez-Landaverde et al. 2005), aldehydes (Vazquez-Landaverde et al. 2005), and sulfides (Vazquez-Landaverde et al. 2006).

In general, the amount of volatile compounds in milk is low and a concentration step is required to perform effective analyses. Dynamic headspace sampling (DHS) in combination with chromatographic techniques is a useful method for concentrating and analyzing volatiles with high reproducibility and sensitivity. Optimally, the volatiles should be collected under mild conditions to minimize sample manipulation and the potential for artifact formation (Dirinck et al. 1984). DHS in combination with gas chromatography-mass spectrometry (GC-MS) has been frequently used for characterizing the volatiles in milk (Valero et al. 2001; Tosó et al. 2002; Vallejo-Cordoba et al. 1993) and is also used in the characterization of many other food matrices (Jensen et al. 2011; Bach et al. 2012). However, to our knowledge, no characterization of the volatile profile of heat-treated lactose-hydrolyzed milk obtained using the combination of DHS and GC-MS has been reported.

The aim of the present study was to identify and compare the volatile fractions of different types of commercially produced enzymatically hydrolyzed UHT milk (direct versus indirect) and conventional UHT milk. To ensure a representative pool of samples, several batches of milk were produced in a full-scale commercial dairy plant, which made it possible also to elucidate batch variations in industrially produced milks.

2 Materials and methods

2.1 Chemical references

Decane (99%), tetradecane (99%), dodecane (99%), 2-methylbutanal (95%), hexanal (98%), octanal (99%), nonanal (95%), decanal (98%), 2-butanone (99%), 2-pentanone
(99%), 2-hexanone (99.5%), 2-heptanone (98%), 2-octanone (98%), 2-nonanone (99%), 6-methyl-5-heptene-2-one (99%), 6,10-dimethyl-5,4-undecadien-2-one (97%), dimethyl disulfide (98%), 4-methyl-1-pentanol (97%), toluene (99.5%), styrene (99%), and 2-pentyl furan (97%) were obtained from Sigma-Aldrich Inc. (Steinheim, Germany). Dimethyl sulfoxide (99%) was purchased from Serva electrophoresis GmbH (Heidelberg, Germany).

2.2 Milk samples

Milk was produced at a Danish commercial dairy plant (Arla Foods, Esbjerg, Denmark). A total of four types of milk (CONVI, LHD, LHI, and FILTI) were produced (Table 1) and stored at 22 °C until the day of analysis. CONVI, LHD, and LHI were produced in three batches in September and October 2012 and February 2013. For technical reasons, FILTI was included to separate the impact of filtration and enzyme treatment on the formation of volatiles. FILTI was only produced in two batches in September 2012. The milk was UHT-treated using either direct heat treatment (LHD) (steam is injected into the milk to heat it to 140 – 145 °C for a few seconds, followed by flash cooling; Tetra Pak 2003) or indirect heat treatment (CONVI, LHI, FILTI) (a tubular heat exchanger is used to heat the milk to 140 – 145 °C for a few seconds, followed by slow cooling (Tetra Pak 2003)) and packaged aseptically. Lactose in the lactose-hydrolyzed and the filtered milk (LHD, LHI, FILTI) was removed using ultrafiltration, to approximately 60% of the original lactose level. The remaining lactose was enzymatically hydrolyzed (LHD, LHI) by the addition of β-galactosidase (Jelen and Tossavainen 2003). A total of three replicates of each milk type and each batch were analyzed 17–21 days after production.

2.3 Dynamic headspace sampling

A 100-mL milk sample was placed in a conical flask with a glass cap insert and 200 μL of a 10 ppm 4-methyl-1-pentanol solution was added to the milk as an internal standard (IS). Adsorbent traps (Tenax TA, 200 g Tenax TA, 35/60 mesh; Markes International Limited, Llantrisant, UK) were attached to the flask, which was subsequently placed in

<table>
<thead>
<tr>
<th>Milk</th>
<th>No. of batches</th>
<th>Enzymatic hydrolysis</th>
<th>Heat treatment</th>
<th>Filtration</th>
<th>Level of lactose (% of original level)</th>
<th>Amount of lactose (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONVI</td>
<td>3</td>
<td>−</td>
<td>Indirect</td>
<td>−</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>LHD</td>
<td>3</td>
<td>+</td>
<td>Direct</td>
<td>+</td>
<td>&lt;1</td>
<td>0.01</td>
</tr>
<tr>
<td>LHI</td>
<td>3</td>
<td>+</td>
<td>Indirect</td>
<td>+</td>
<td>&lt;1</td>
<td>0.01</td>
</tr>
<tr>
<td>FILTI</td>
<td>2</td>
<td>−</td>
<td>Indirect</td>
<td>+</td>
<td>60</td>
<td>3</td>
</tr>
</tbody>
</table>
a thermostatically controlled cabinet at 20 °C. The milk was sparged with nitrogen (40 mL.min) for 45 min while being stirred.

2.4 GC-MS

The adsorbent traps were desorbed (250 °C, 15 min) into a cold trap (U-T11GCP, Markes International Limited, UK) using a thermal desorption system (ultra-UNITY™, Markes International Limited, Llantrisant, UK). Helium was used as carrier gas at a constant pressure of 6.985×10³ Pa. The desorbed volatiles were focused at 1 °C for 5 min and then injected onto a gas chromatography column through a transfer line (140 °C) by raising the temperature of the trap to 300 °C under splitless conditions. The gas chromatograph (Finnigan trace GC ultra, Thermo, Waltham, MA) was equipped with a CP-Wax 52CB (Varian, Palo Alto, CA, USA) column (50 m×0.25 mm, film thickness 0.25 μm). The gradient program included an isothermal step at 32 °C for 1 min, a ramp to 35 °C at 0.2 °C.min, a ramp to 40 °C at 0.5 °C.min, a ramp to 60 °C at 2.5 °C.min, and a ramp to 220 °C at 10 °C.min, followed by a 15-min isothermal step.

The GC was connected to a single quadrupole mass spectrometer (Finnigan Trace dual-stage quadrupole (DSQ), Thermo, Waltham, MA, USA). The MS transfer line temperature was 250 °C and the ion source temperature was 200 °C. The mass spectrometer was scanned over a mass to charge (m/z) range of 45–650 (4.46 scans/s) and the spectra were obtained using a fragmentation voltage of 70 eV.

2.5 Method optimization

To optimize the sampling parameters, the effect of flow, sample volume, and headspace sampling time were initially studied. Final sampling parameter values were set to 40 mL.min, 100 mL, and 45 min, respectively. The sampling temperature was fixed at 20 °C. In addition, several trap and cold trap materials were tested, and the injection mode and GC temperature gradient were adapted to obtain optimal separation of the volatile compounds. The final trap material used was Tenax TA and the coldtrap material was U-T11GPC. The optimum parameter values were selected on the basis of peak shape, separation, intensity, and sensitivity.

2.6 Identification and quantitative analysis

A MS database (NIST MS search version 2.0, 2011) was used to identify 24 volatile compounds. In addition, all of the compounds except 3,3-dimethyl hexane, 2-undecanone, and dimethyl trisulfide were verified by comparison of mass spectral data and retention times with authentic reference compounds.

The identified volatile compounds were quantified using external standard curves prepared in duplicates. The four external calibrants, which were eluted in different regions of the chromatogram, were 2-pentanone (eluting at 10.7 min), hexanal (19.2 min), 2-heptanone (31.9 min), and decanal (eluting at 44.5 min). Analytes eluting in the same retention time window were assumed to have comparable response coefficient and equal linear range as the calibrant (Jensen et al. 2011). However, the ketone responses reminded more of the nearest ketone external calibrant and were quantified (μg.kg milk) according to the appropriate calibrant regression equation.
The regression equation, limits of detection (LOD), and limits of quantification (LOQ) were calculated from the standard error of the y-intercept and the slope of the linear regression, for each standard curve (Table 2) (Dolan 2009a, b).

2.7 Multivariate data and statistical analysis

Multivariate data analysis in the form of principal component analysis (PCA) was applied including all quantified compounds to investigate grouping of the samples using the Simca software (version 13.0, Umetrics AB, Umeå, Sweden). Data were normalized against the area of the added IS and unit-variance (UV) scaled before PCA analysis.

To identify significant differences between the milk samples, univariate statistical analysis including linear mixed model and Tukey’s honest significant difference ($\alpha = 0.05$) test was performed using the software R (version 2.15.2, R Foundation for Statistical Computing, Vienna, Austria). The variance-covariance was estimated using restricted maximum likelihood (REML) to avoid underestimations of random variations in the data. The statistical model (linear mixed effect, LME) included fixed factors for the four milk types (CONVI, LHD, LHI, FILTI) and the 11 batches and took into account the correlation between the three replicates from the same batch. The variance within milk types was estimated using a linear model and Tukey’s honest significant difference ($\alpha = 0.05$), and Bonferroni’s method was used to maintain the family-wise error rate (FWER). Compounds below the LOD and LOQ were not included in the statistical analysis.

3 Results

GC-MS analyses were conducted on the 11 different industrially produced milk batches included in the study. Representative total ion current (TIC) chromatograms obtained from conventional, lactose-hydrolyzed direct and indirect treated UHT milk, and filtered UHT milk are shown in Fig. 1. A total of 24 volatile compounds including aldehydes, ketones, hydrocarbons, and sulfides were identified and quantified in the milk samples (Table 3). A comparison of the chromatograms indicates that the conventional UHT milk contains a greater amount of the ketones 2-butanone (7.61 min), 2-pentanone (10.6 min), 2-heptanone (31.91 min), 2-nonanone (42.04 min), and 2-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calibration concentrations (µg.kg)</th>
<th>Limit of detection (LOD) (µg.kg)</th>
<th>Limit of quantification (LOQ) (µg.kg)</th>
<th>Regression equation</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Pentanone</td>
<td>4.0–79.4</td>
<td>3.8</td>
<td>11.6</td>
<td>$y=0.198x$</td>
<td>0.997</td>
</tr>
<tr>
<td>Hexanal</td>
<td>4.0–31.8</td>
<td>2.0</td>
<td>6.2</td>
<td>$y=0.2295x+0.1999$</td>
<td>0.994</td>
</tr>
<tr>
<td>2-Heptanone</td>
<td>6.4–64.3</td>
<td>9.4</td>
<td>28.4</td>
<td>$y=0.2762x$</td>
<td>0.985</td>
</tr>
<tr>
<td>Decanal</td>
<td>16.3–244.1</td>
<td>13.9</td>
<td>42.1</td>
<td>$y=0.0108x+0.0711$</td>
<td>0.994</td>
</tr>
</tbody>
</table>
undecanone (46.2 min) compared to lactose-hydrolyzed milks (Fig. 1a–e). In addition, a batch-to-batch variation was observed for the ketones when two batches of direct treated lactose-hydrolyzed (Fig. 1b, c) and indirect treated UHT milk (Fig. 1d, e) were compared.

PCA was carried out on the 24 volatile compounds identified, and Fig. 2 shows the PCA score scatter plot (a) and the corresponding loading plot (b) of the volatile compounds identified in the milk types. In the score plot, principal components (PC) 1 and 2 describe 38% and 23.6% of the variation, respectively. The score plot also indicates a separation of the lactose-hydrolyzed samples along PC1 and filtered samples along PC2. However, the separation of the lactose-hydrolyzed milk away from the lactose-containing milk is batch dependent, and one batch each of LHD and LHI was more similar to the CONVI milk. Our data therefore exhibit a significant batch variation which tends to be more severe for lactose-hydrolyzed milk than for conventional milk. The loading scatter plot (Fig. 2b) reveals that the separation of the milk types along PC1 may be ascribed to differences in the concentration of ketones and the aldehydes octanal, nonanal, and decanal, while PC2 reveals differences in the concentration of the sulfides, dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and dimethyl sulfoxide (DMSO).

It was observed that CONVI tended to contain a greater amount of ketones than LHD or LHI milks, which in contrast were characterized by a higher content of the aldehydes nonanal and decanal, if the two outlier milk batches LHD (batch 1) and LHI (batch 3) were excluded. In general, no clear difference was seen between direct and
Table 3  Average concentrations [μg.kg] of volatile compounds in conventional, lactose-hydrolyzed, and filtered UHT milk

<table>
<thead>
<tr>
<th>Rt [min]</th>
<th>Volatile compound</th>
<th>Conventional indirect UHT (CONVI)</th>
<th>Lactose-hydrolyzed direct UHT (LHD)</th>
<th>Lactose-hydrolyzed indirect UHT (LHI)</th>
<th>Filtered UHT (FILTI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.06</td>
<td>3,3-Dimethylhexane</td>
<td>15.59a 8.86b 20.89b</td>
<td>19.01a 14.38b 21.47c</td>
<td>38.52a 30.16b 23.58c</td>
<td>31.70 2.51*</td>
</tr>
<tr>
<td>12.23</td>
<td>Decane</td>
<td>0.51* 0.55* 3.50*</td>
<td>1.27* 1.21* 0.72*</td>
<td>0.96* 2.19* 1.65*</td>
<td>0.27* 0.77*</td>
</tr>
<tr>
<td>34.50</td>
<td>Dodecane</td>
<td>0.01* nd 0.88*</td>
<td>&lt;0.01* 0.12* 0.71*</td>
<td>0.52* 0.73* 1.60*</td>
<td>0.98* 2.45*</td>
</tr>
<tr>
<td>42.43</td>
<td>Tetradecane</td>
<td>nd nd nd</td>
<td>nd nd nd</td>
<td>nd nd nd</td>
<td>10.51* 42.73</td>
</tr>
<tr>
<td></td>
<td>Total content of alkanes</td>
<td>16.11 9.41 25.27</td>
<td>20.29 15.71 22.90</td>
<td>40.00 33.08 26.83</td>
<td>43.46 48.46</td>
</tr>
<tr>
<td>8.85</td>
<td>2-Methylbutanal</td>
<td>0.06* 0.27* 0.48*</td>
<td>0.05* nd nd</td>
<td>nd nd 0.18* nd</td>
<td>nd nd</td>
</tr>
<tr>
<td>19.23</td>
<td>Hexanal</td>
<td>0.02* nd 2.59#</td>
<td>nd nd nd</td>
<td>nd nd 1.67* nd</td>
<td>0.45* 1.13*</td>
</tr>
<tr>
<td>39.01</td>
<td>Octanal</td>
<td>nd nd nd</td>
<td>nd nd nd</td>
<td>nd nd nd</td>
<td>0.97* 4.03*</td>
</tr>
<tr>
<td>42.17</td>
<td>Nonanal</td>
<td>1.04* 1.35* nd</td>
<td>0.73* 5.71* nd</td>
<td>5.29* 9.83* nd</td>
<td>13.50* 6.49*</td>
</tr>
<tr>
<td>44.49</td>
<td>Decanal</td>
<td>nd nd nd</td>
<td>nd 4.20* nd</td>
<td>0.4* 3.30* nd</td>
<td>nd 3.75*</td>
</tr>
<tr>
<td></td>
<td>Total content of aldehydes</td>
<td>1.12 1.62 3.07</td>
<td>0.78 9.91 nd</td>
<td>5.69 13.13 1.85</td>
<td>14.92 15.40</td>
</tr>
<tr>
<td>7.61</td>
<td>2-Butanone</td>
<td>91.43a 73.90b 105.10c</td>
<td>49.13 7.06a 9.62a</td>
<td>6.35a 6.20a 99.11</td>
<td>57.60a 50.50b</td>
</tr>
<tr>
<td>10.73</td>
<td>2-Pentanone</td>
<td>21.67a 34.51b 24.25c</td>
<td>15.47 5.36a 5.68a</td>
<td>4.61a 4.58a 20.82</td>
<td>17.79a 17.39a</td>
</tr>
<tr>
<td>18.95</td>
<td>2-Hexanone</td>
<td>&lt;0.01* 0.13* 0.73*</td>
<td>nd nd nd</td>
<td>nd nd nd</td>
<td>0.25* nd</td>
</tr>
<tr>
<td>31.91</td>
<td>2-Heptanone</td>
<td>56.03a 93.98b 76.09c</td>
<td>49.20a 15.60a 23.47b</td>
<td>14.73a 14.05a 90.64</td>
<td>45.96a 45.30a</td>
</tr>
<tr>
<td>38.93</td>
<td>2-Octanone</td>
<td>0.17* 0.38* 0.47*</td>
<td>0.23* 0.04* 0.01*</td>
<td>0.04* 0.05* 0.19*</td>
<td>0.26* 0.39*</td>
</tr>
<tr>
<td>40.65</td>
<td>6-Methyl-5-hepten-2-one</td>
<td>0.34* nd nd</td>
<td>nd 0.33* nd</td>
<td>0.26* 0.44* nd</td>
<td>0.23* 0.74*</td>
</tr>
</tbody>
</table>
Table 3 (continued)

<table>
<thead>
<tr>
<th>Rt [min]</th>
<th>Volatile compound</th>
<th>Conventional indirect UHT (CONVI)</th>
<th>Lactose-hydrolyzed direct UHT (LHD)</th>
<th>Lactose-hydrolyzed indirect UHT (LHI)</th>
<th>Filtered UHT (FILTI)</th>
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<tr>
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<td></td>
<td>1 2 3</td>
<td>1 2 3</td>
<td>1 2 3</td>
<td>1 2</td>
</tr>
<tr>
<td>42.04</td>
<td>2-Nonanone</td>
<td>10.68&lt;sup&gt;a&lt;/sup&gt; 17.12&lt;sup&gt;b&lt;/sup&gt; 11.68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.55 2.86&lt;sup&gt;<em>&lt;/sup&gt; 3.57&lt;sup&gt;</em>&lt;/sup&gt;</td>
<td>3.02&lt;sup&gt;<em>&lt;/sup&gt; 2.93&lt;sup&gt;</em>&lt;/sup&gt; 14.35</td>
<td>11.09&lt;sup&gt;a&lt;/sup&gt; 11.46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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<td>46.19</td>
<td>2-Undecanone</td>
<td>0.92&lt;sup&gt;<em>&lt;/sup&gt; 1.94&lt;sup&gt;</em>&lt;/sup&gt; 1.10&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.90&lt;sup&gt;<em>&lt;/sup&gt; 0.11&lt;sup&gt;#&lt;/sup&gt; 0.09&lt;sup&gt;</em>&lt;/sup&gt;</td>
<td>0.19&lt;sup&gt;<em>&lt;/sup&gt; 0.22&lt;sup&gt;</em>&lt;/sup&gt; 1.33&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.16&lt;sup&gt;<em>&lt;/sup&gt; 1.32&lt;sup&gt;</em>&lt;/sup&gt;</td>
</tr>
<tr>
<td>49.71</td>
<td>6,10-Dimethyl-5,9-undecadien-2-one (E)</td>
<td>0.09&lt;sup&gt;*&lt;/sup&gt; nd nd</td>
<td>nd nd nd</td>
<td>nd nd 0.10&lt;sup&gt;*&lt;/sup&gt;</td>
<td>nd nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total content of ketones</td>
<td>181.34 221.96 219.42</td>
<td>124.48 31.36 42.44</td>
<td>29.2 28.47 226.79</td>
</tr>
<tr>
<td>17.82</td>
<td>Dimethyl disulfide</td>
<td>8.70&lt;sup&gt;a&lt;/sup&gt; 5.48&lt;sup&gt;##&lt;/sup&gt; 13.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.92&lt;sup&gt;##&lt;/sup&gt; 6.37&lt;sup&gt;a&lt;/sup&gt; 7.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.72 4.83&lt;sup&gt;##&lt;/sup&gt; 5.70&lt;sup&gt;##&lt;/sup&gt;</td>
<td>3.94&lt;sup&gt;##&lt;/sup&gt; 4.11&lt;sup&gt;##&lt;/sup&gt;</td>
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<tr>
<td>41.67</td>
<td>Dimethyl trisulfide</td>
<td>73.06&lt;sup&gt;a&lt;/sup&gt; 51.74&lt;sup&gt;b&lt;/sup&gt; 136.74&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.91&lt;sup&gt;##&lt;/sup&gt; 73.17&lt;sup&gt;a&lt;/sup&gt; 77.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.42&lt;sup&gt;a&lt;/sup&gt; 54.91&lt;sup&gt;a&lt;/sup&gt; 33.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.94&lt;sup&gt;a&lt;/sup&gt; 32.17&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>46.07</td>
<td>Dimethyl sulfoxide</td>
<td>7.24&lt;sup&gt;##&lt;/sup&gt; 20.44&lt;sup&gt;##&lt;/sup&gt; 13.99&lt;sup&gt;##&lt;/sup&gt;</td>
<td>5.30&lt;sup&gt;##&lt;/sup&gt; nd nd</td>
<td>nd nd 1.35&lt;sup&gt;##&lt;/sup&gt;</td>
<td>12.22&lt;sup&gt;##&lt;/sup&gt; 41.63&lt;sup&gt;##&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total content of sulfides</td>
<td>89.00 77.66 161.14</td>
<td>35.13 79.54 85.75</td>
<td>63.14 59.74 40.75</td>
</tr>
<tr>
<td>14.78</td>
<td>Toluene</td>
<td>4.91&lt;sup&gt;##&lt;/sup&gt; 4.71&lt;sup&gt;##&lt;/sup&gt; 8.14</td>
<td>12.44 1.91&lt;sup&gt;<em>&lt;/sup&gt; 1.74&lt;sup&gt;</em>&lt;/sup&gt;</td>
<td>2.44&lt;sup&gt;##&lt;/sup&gt; 2.55&lt;sup&gt;##&lt;/sup&gt; 5.92&lt;sup&gt;##&lt;/sup&gt;</td>
<td>12.30&lt;sup&gt;##&lt;/sup&gt; 12.53&lt;sup&gt;##&lt;/sup&gt;</td>
</tr>
<tr>
<td>36.33</td>
<td>2-Pentylfuran</td>
<td>0.32&lt;sup&gt;<em>&lt;/sup&gt; 0.59&lt;sup&gt;</em>&lt;/sup&gt; 0.79&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.44&lt;sup&gt;<em>&lt;/sup&gt; 0.14&lt;sup&gt;</em>&lt;/sup&gt; &lt;0.01&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.14&lt;sup&gt;<em>&lt;/sup&gt; 0.21&lt;sup&gt;</em>&lt;/sup&gt; 0.17&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.53&lt;sup&gt;<em>&lt;/sup&gt; 0.93&lt;sup&gt;</em>&lt;/sup&gt;</td>
</tr>
<tr>
<td>37.34</td>
<td>Styrene</td>
<td>1.69&lt;sup&gt;<em>&lt;/sup&gt; 1.25&lt;sup&gt;</em>&lt;/sup&gt; 1.80&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.40&lt;sup&gt;<em>&lt;/sup&gt; 0.48&lt;sup&gt;</em>&lt;/sup&gt; 0.07&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.63&lt;sup&gt;<em>&lt;/sup&gt; 0.59&lt;sup&gt;</em>&lt;/sup&gt; 0.46&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.99&lt;sup&gt;<em>&lt;/sup&gt; 0.43&lt;sup&gt;</em>&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Numbers 1, 2, and 3 represent the batch number for each milk type (three replicates for each batch). Superscript letters (a-c) indicate significance (P<0.05) within the milk types. <sup>nd</sup> not detected, <sup>*</sup> concentration <LOD, <sup>##</sup> concentration <LOQ.
indirect lactose-hydrolyzed UHT (milk types LHD and LHI). The score plot in Fig. 2a partly places lactose-reduced, filtered UHT milk (FILTI) between the CONVI milk type and the LHD and LHI milk types, which may be ascribed to differences in ketones and the aldehydes nonanal and decanal (Fig. 2b). In contrast, FILTI is separated from the other milk types due to increased concentrations of tetradecane, DMSO, and 2-pentyl furan.

The volatile compounds were quantified based on the four calibration curves (Table 2). The concentrations of the 24 volatile compounds in the milk types are summarized in Table 3. However, some of the compounds were below the LOQ, and therefore, the given concentrations are only tentatively quantified. The volatile compounds varied widely in concentration within batches of similar milk types, which was visually seen in the PCA score scatter plot in Fig. 2a. Generally, ketones represented the chemical class with the highest number of detected compounds. In total, nine ketones were identified, and among these, 2-butanone, 2-heptanone, and 2-nonanone were present in the highest concentrations. These three methyl ketones were also the compounds that differed most in concentration between CONVI, LHD, and LHI according to the TIC in Fig. 1. In total, five aldehydes were identified, and of these, nonanal was the compound found in the highest concentration in all of the milk types analyzed. Of the three sulfides identified (DMDS, DMTS, DMSO), DMTS was found

**Fig. 2** a PCA score plot with principal components 1 and 2, describing 38 % and 23.6 % of the total variation in the volatile compound profiles, respectively. Circle A: Conventional indirect UHT milk, square B: lactose-hydrolyzed direct UHT milk, triangle C: lactose-hydrolyzed indirect UHT milk, inverted triangle D: Filtered indirect UHT milk, numbers 1–3 indicate batch number. b Corresponding loading plot. $R^2=0.739$ and $Q^2=0.492$, with three components.
to be present in the highest concentration in all milk types. Overall, no significant differences could be identified between the milk types (CONVI, LHD, LHI, or FILTI) for any of the detected volatile compounds. However, significant differences within milk types were observed due to high batch-to-batch variation in the industrially produced samples (Table 3, Fig. 2a).

4 Discussion

Reports on the volatile profile of lactose-hydrolyzed milk are relatively sparse (Jelen and Tossavainen 2003; Kallioinen and Tossavainen 2008; Messia et al. 2007; Tossavainen and Kallioinen 2007). DHS and thermal desorption combined with GC-MS is a useful and efficient method for collecting and analyzing volatiles (Naudé et al. 2009). In the present study, this method combined with multivariate data analyses was applied to obtain volatile profiles of lactose-hydrolyzed, filtered, and conventional UHT-treated milk. To our knowledge, no elucidation of the volatile profile of heat-treated lactose-hydrolyzed milk obtained using the combination of DHS and GC-MS has previously been reported.

A total of 24 volatile compounds were identified and quantified (Table 3). The number of ketones and aldehydes identified is in line with the results obtained by Vazquez-Landaverde et al. (2005), who identified eight ketones and nine aldehydes when investigating volatile compounds in UHT milk using headspace solid-phase microextraction GC. The 24 quantified volatile compounds (Table 3) ranged in concentration from <0.01 to 105.10 μg.kg milk. The concentrations of the high-concentration compounds in the present study were somewhat higher, and the concentrations of the low-concentration compounds were lower than previously reported (Valero et al. 2001; Vazquez-Landaverde et al. 2005; Toso et al. 2002; Contarini et al. 1997; Vazquez-Landaverde et al. 2006; Al-Attabi 2009). This divergence in results is most probably due to differences in analytical and quantification procedures but also in the processing of the milk (e.g., heat treatment and packaging).

It is known that aldehydes and ketones contribute to the “stale” note found in UHT milk (Rerkrai et al. 1987; Vazquez-Landaverde et al. 2005) and this becomes more evident when the “cooked” flavor begins to decline (Thomas et al. 1975). PCA indicated that the secondary lipid peroxidation products octanal and nonanal were important for the differentiation of LHI and FILTI milks from the other milks (Fig. 2). However, the levels of octanal and nonanal were below LOD (Table 3), and further studies are therefore needed to substantiate this finding. The presence of the aldehydes is an indicator of lipid oxidation in the milk, and Vazquez-Landaverde et al. (2005) reported that although the aldehydes were less affected by heat treatment in comparison with ketones, an increase in the total concentration of aldehydes increased with the severity of heat treatment.

Ketones were the most abundant volatile compounds in all of the milk types in accordance with previous findings in conventional UHT milk (Vazquez-Landaverde et al. 2005; Contarini and Povolo 2002; Contarini et al. 1997). In general, the present study indicates that conventional UHT milk contained higher concentrations of ketones than lactose-hydrolyzed or filtered milk (Fig. 2a, Table 3).

The methyl ketones 2-butanone, 2-heptanone, and 2-nonanone were among the ketones present in the highest concentrations in both conventional and lactose-
hydrolyzed milk. This is consistent with the results of Vazquez-Landaverde et al. (2005), who concluded that 2-heptanone and 2-nonanone had a major impact on the flavor of heat-treated milk. In heat-treated milk, ketones are mainly products of the heat-initiated decarboxylation of β-oxidized saturated fatty acids or decarboxylation of β-ketoacids (Vazquez-Landaverde et al. 2005; Belitz et al. 2004). In general, ketone formation requires a low activation energy (Schwartz et al. 1966), and therefore, the production of these compounds progresses throughout the storage period following initiation of heat-induced oxidation (Kochhar 1996). As a result of a marked batch-to-batch variation in the milks, no significant differences in the content of ketones between the different milk types were evident. Intriguingly, 2-methylbutanal, a characteristic Maillard reaction product, was detected in all three batches of conventional UHT milk but only sporadically seen in the lactose-hydrolyzed milk. 2-Methylbutanal is a Strecker degradation product of isoleucine and is an important indicator for this reaction having taken place in severely heat-treated milk (Belitz et al. 2004). Adamiec et al. (2001) suggested that Strecker degradation of amino acids includes radical reactions, and more radicals may have been produced in the conventional UHT milk than in the lactose-hydrolyzed UHT milk based on the higher concentration of 2-methylbutanal in the former. The fact that Maillard reaction products formed in the final stage are well-known antioxidants (Yilmaz and Toledo 2005) and that the Maillard reaction proceeds more extensively in glucose- and galactose-protein model systems (Kato et al. 1986, 1988) may explain a more limited heat-activated lipid oxidation (lower ketone concentration). Furthermore, the presence of antioxidants may have affected the formation of 2-methylbutanal in lactose-hydrolyzed UHT milk, considering that this compound may be formed through a radical process as outlined above.

Three sulfide compounds were identified in the present study. Sampling and analysis of sulfides is difficult because of their high volatility and reactivity, which also explains the large variation in the concentration of these compounds (Table 3). The concentration of sulfides increases with the severity of heat treatment, primarily due to denaturation of β-lactoglobulin (Hutton and Patton 1952), which exposes the sulfur-containing amino acids cysteine and methionine to oxidation or Strecker degradation (Hutton and Patton 1952; Datta et al. 2002). DMDS and DMTS are products of Strecker degradation of methionine, and DMSO is formed when the Strecker degradation product dimethyl sulfide (DMS) is oxidized (Al-Attabi et al. 2008). The presence of sulfides in milk is an indication of Maillard reaction, and the compounds are characteristic for the flavor formation of UHT milk (Al-Attabi 2009). Surprisingly, DMS, which previously was found to be the most abundant sulfide in milk (Vazquez-Landaverde et al. 2006), was not identified in the present study, probably due to the high volatility of the compound, the mild collection conditions, and inadequate sensitivity of the method for sulfide compounds.

Four alkanes were identified in the milk (Table 3), and higher concentrations of 3,3-dimethylhexane and tetradecane were observed in LHI and FILTI compared with UHT and LHD. The origin of alkanes in milk is far from well-understood (Coppa et al. 2011); however, the compounds may be formed from fatty acid oxidation and thermal degradation of amino acids (Lien and Nawar 1973; Lien and Nawar 1974; Macku and Takayuki 1991; Whitfield 1992). There are no obvious explanations why a difference in alkane concentration was observed between the
different milk types in the present study. However, these compounds were considered to have a minor impact on the volatile profile of the different milk types.

Previous studies have reported that direct UHT treatment represents a less severe process than indirect UHT treatment (Badings et al. 1981) due to the shorter heating and cooling times. Perkins et al. (2005) found that the amount of ketones was approximately two times higher in 1-week-old indirect UHT-treated milk than in direct UHT-treated milk. Badings et al. (1981) found that the compounds primarily responsible for the differences between the two heat-treatment procedures were not only 2-heptanone and 2-nonanone, but also DMDS, 2-hexanone, 2-octanone, and 2-undecanone. In the present study, there were no clear differences in the volatile compound profiles between the two lactose-hydrolyzed milk types LHD and LHI, suggesting that the direct and indirect heat treatment do not have the same effect on lactose-hydrolyzed UHT milk as previously observed for conventional UHT milk. However, the volatile profile of the milk types can be expected to develop differently during storage (Perkins et al. 2005), an aspect which was not investigated in the present study.

Lactose-hydrolyzed milk is an alternative to conventional milk, and therefore, the dairy industry strives to produce lactose-hydrolyzed milk with characteristics resembling conventional milk. However, the present study reveals that considerable product differences between milk batches exist, which may impair the consumers’ recognition of the product. Lactose-hydrolyzed milk produced through partial physical removal of lactose and subsequent enzymatic hydrolysis of the remaining lactose is a rather new product, and a full understanding of its quality characteristics is important to ensure consumer acceptance. Consequently, the grouping of conventional, filtered, and lactose-hydrolyzed UHT milk in Fig. 2 is highly relevant in relation to optimizing the flavor profile of lactose-hydrolyzed milk.

Moreover, significant batch variations were present in all types of milk which is deemed to be a challenge in the dairy industry. Consumers are relatively sensitive to variations in flavor (Thomas 1981). The batch variation in two out of six batches of the lactose-hydrolyzed milk was due to differences in the ketone concentrations. This may be ascribed to a number of factors, including seasonal variations in the raw milk (Auldist et al. 1998; Ostersen et al. 1997) and differences in processing. The latter seems most feasible as industrial-scale thermal processing can subject certain batches of milk to a more severe heat load. This would explain the difference in ketone concentration, as a higher ketone concentration may result from more severe heat treatment (Vazquez-Landaverde et al. 2005; Contarini and Povolo 2002; Contarini et al. 1997). Similar variations in ketone concentration between batches and brands have been noted in previous studies by Perkins et al. (2005) and Vazquez-Landaverde et al. (2005).

5 Conclusion

The present study focused on the analysis of short-term stored lactose-hydrolyzed and conventional UHT milk, being analyzed 2–3 weeks after production at the dairy plant. GC-MS analysis indicated that the content of ketones in general was higher in conventional UHT milk than in lactose-hydrolyzed UHT milk; however, no significant differences in the volatile compound profiles could be observed between the milk types.
as a result of a batch-to-batch variation. However, it is hypothesized that the volatile profile of conventional and lactose-hydrolyzed UHT milk will change during storage and that differences in the volatile profiles of the milk will increase during extended storage. Further studies are necessary to obtain a comprehensive understanding of how the volatile profile of lactose-hydrolyzed milk will change during storage, and how these changes affect the quality of the milk.

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Paper 2

Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk


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Lactose-Hydrolyzed Milk Is More Prone to Chemical Changes during Storage than Conventional Ultra-High-Temperature (UHT) Milk

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ABSTRACT: The enzymatic hydrolysis of lactose to glucose and galactose gives rise to reactions that change the chemistry and quality of ambient-stored lactose-hydrolyzed ultra-high-temperature (UHT) milk. The aim of the present study was to investigate and compare chemical changes in lactose-hydrolyzed and conventional UHT milk during a 9 month ambient storage period. Several complementary analyses of volatiles, free amino acids, acetate, furosine, and level of free amino terminals were concluded. The analyses revealed an increased level of free amino acids and an increased formation rate of specific compounds such as furosine and 2-methylbutanal in lactose-hydrolyzed UHT milk compared to conventional UHT milk during storage. These observations indicate more favorable conditions for Maillard and subsequent reactions in lactose-hydrolyzed milk compared to conventional UHT milk stored at ambient temperature. Furthermore, it is postulated that proteolytic activity from the lactase-enzyme preparation may be responsible for the observed higher levels of free amino acids in lactose-hydrolyzed UHT milk.

KEYWORDS: lactose-hydrolyzed milk, aldehydes, heat-induced changes, Maillard reaction, furosine, long-term storage

INTRODUCTION

Ultra-high-temperature (UHT) treatment and subsequent ambient storage of milk have been studied extensively. The high temperature (130–150 °C, 2–10 s) destroys bacteria and inactivates most of the enzymes present in milk, and thereby the milk shelf life is extended up to 8 months at ambient temperature. However, the severe heat treatment also causes protein denaturation and nonenzymatic reaction building (Maillard reactions) and initiates oxidative processes that deteriorate the quality of UHT products during storage. This deterioration can result in quality defects such as color change, flavor change, and gelation that shorten the shelf life of UHT products.

As the result of an increased awareness of lactose intolerance, new dairy products without lactose have become commercially available in the market. Lactose-hydrolyzed (LH) milk may be produced using ultra- and nanofiltration, which removes approximately 40% of the lactose, and enzymatic hydrolysis by the addition of lactase. The net result is milk with approximately 60% of the original carbohydrate content, composed of the reducing sugars glucose (approximately 2.5 g/100 mL milk) and galactose (approximately 2.5 g/100 mL milk) instead of lactose. Studies have indicated that lactose-hydrolyzed milk may be more vulnerable to chemical changes during processing and storage than conventional milk. Additionally, the added lactase enzyme may give rise to unwanted side activities, for example, proteolytic activity.

In an effort to better understand the reactions that take place in lactose-hydrolyzed milk during storage and how these reactions affect the subsequent milk characteristics, the sensory characteristics, proteolytic activity, and role of heat load have all been studied in detail. Higher levels of cooked and processed flavor, proteolytic activity, and furosine were observed in lactose-hydrolyzed UHT milk compared to conventional UHT milk. The Maillard reactions have been reported to form as many as 3500 different volatile compounds, including aldehydes, ketones, and sulide compounds. Some of these volatile compounds are formed in lactose-hydrolyzed UHT milk during heating and may affect the flavor and quality of the corresponding milk during storage, for example, by shortening its shelf life compared to the shelf life of conventional UHT milk. Thus, it is of fundamental importance to investigate the changes in volatile composition over the entire shelf life of this type of milk, so that the production of lactose-hydrolyzed milk can be better understood in relation to shelf life extending initiatives. The aim of the present study was to elucidate the detailed chemical changes in enzymatically lactose-hydrolyzed milk (both direct and indirect UHT-treated) and conventional UHT milk during a 9 month storage period. To support the hypothesis that lactose-hydrolyzed UHT milk is more
susceptible to Maillard induced chemical changes, proteolytic and other non-Maillard chemical changes were observed during storage compared to corresponding changes in conventional UHT milk.

**MATERIAL AND METHODS**

**Chemical References.** Decane (99%), tetradecane (99%), dodecane (99%), 2-methylbutan-1-ol (95%), hexanol (98%), heptanal (95%), 2-octanone (99%), furfural (98%), nonanal (95%), decanal (98%), 2-butanone (99%), 2-pentanone (99%), 2-hexanone (99.5%), 2-heptanone (98%), 2-octanone (98%), 2-nonanone (99%), 2-methyl-5-heptene-2-one (99%), 6,10-dimethyl-5,4,8-tridecadien-2-one (97%), 2-undecanone (99%), dimethyl disulfide (98%), dimethyl trisulfide (98%) 4-methyl-1-pentanol (97%), toluene (99.5%), styrene (99%), 2-methylfuran (98%), 2-ethylfuran (99%), 2-pentylfuran (97%), fluorescamine (4-phenylspiro[furan-2(3H),1,3-benzisoxazole]-3,3-dione) (99%), and D2O containing 0.025% sodium trimethylsilyl-(2,2,3,3-2H4)-1-propionate were obtained from Sigma-Aldrich Inc. (Steinheim, Germany). Trichloroacetic acid, HCl, and water-free acetone were from Merck (Darmstadt, Germany).

**Milk Samples.** Milk for the study was produced at a commercial Danish dairy plant (Arla Foods, Esbjerg, Denmark) (Figure 1). Three types of milk (conventional indirect UHT (CONVI), lactose-hydrolyzed direct UHT (LHD), and lactose-hydrolyzed indirect UHT (LHI)) were produced (Table 1). The milk was UHT-treated using either direct heat treatment (LHD) (steam is injected into the milk to heat it followed by flash cooling12) (140–145 °C for 2–3 s) or indirect heat treatment (CONVI, LHI) (a tubular heat exchanger is used to heat the milk, followed by slow cooling13) (140–145 °C for 2–3 s) and packaged aseptically in 1 L Tetra Brik cartons. Lactose in the lactose-hydrolyzed milk (LHD, LHI) was removed (approximately 40% of initial lactose content) before heat treatment using ultra- and nanofiltration. The remaining lactose was enzymatically hydrolyzed (LHD, LHI), after heat treatment, by the addition of lactase (Maxilact LAG 5000, DSM, Limburg, The Netherlands).

After transportation to the laboratory, the milk samples were stored in an air-conditioned room at a constant temperature of 22 °C. At days 7, 14, 21, 28, 45, 60, 90, 120, 150, 180, 210, 240, and 270 after production, two milk cartons were picked and frozen and stored at −20 °C until analysis. Milk for the NMR spectroscopy analysis and fluorescamine determination was filtered using Amicon Ultra 0.5 mL 10 kDa (Millipore, Bedford, MA, USA) spin filters at 10000 g for 15 min to remove residual lipids and protein prior to freezing. After frozen storage, the milk samples and filtrates were randomized and thawed in 4 °C before final analyses.

**Determination of Volatile Compounds by Dynamic Headspace Sampling and GC-MS.** The volatile compounds were extracted using dynamic headspace sampling and analyzed by gas chromatography–mass spectrometry (GC-MS) according to the procedure described by Jansson et al.13 The volatile compounds were isolated on adsorbent traps (Tenax TA, 200 g Tenax TA, 35/60 mesh (Markes International Limited, Llantrisant, UK)) and desorbed (250 °C, 15 min) into a cold trap (U-TTIGCP, Markes International Limited) using a thermal desorption system (ultra-UNITY, Markes International Limited) and subsequently injected onto a gas chromatograph (Finnigan Trace GC ultra, Thermo, Waltham, MA, USA) using a CP-Wax 52CB column (50 m × 0.25 mm, film thickness = 0.25 μm (Varian, Palo Alto, CA, USA)).

To identify the separated volatile compounds, a single-quadrupole mass spectrometer (Finnigan Trace dual-stage quadrupole (DSQ), Thermo) was used. The mass spectrometer scanned over a mass to charge (m/z) range of 45–350 (1.61 scans/s) in TIC mode, and specific m/z values were detected in SIM mode (Table 2). The volatile compounds were identified using an MS database (NIST MS search 2.0, 2011) and verified by comparison of mass spectral data and retention times with those of authentic reference compounds.

**Table 1. Overview of Milk Types Included in the Study, Heat Treatments, and Lactose Levels**

<table>
<thead>
<tr>
<th>milk</th>
<th>enzymatic hydrolysis</th>
<th>heat treatment</th>
<th>filtration</th>
<th>level of lactose (% of original level)</th>
<th>amount of lactose (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONVI conventional UHT</td>
<td>–</td>
<td>indirect</td>
<td>–</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>LHD lactose-hydrolyzed direct UHT</td>
<td>+</td>
<td>direct</td>
<td>+</td>
<td>&lt;1</td>
<td>0.01</td>
</tr>
<tr>
<td>LHI lactose-hydrolyzed indirect UHT</td>
<td>+</td>
<td>indirect</td>
<td>+</td>
<td>&lt;1</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*All milks contained 1.5% w/w fat and 3.5% w/w protein.*
A total of 15 identified volatile compounds were quantified using external standard curves prepared in duplicates in UHT milk. For each standard curve, the regression equation, limit of detection (LOD), and limit of quantification (LOQ) were calculated from the standard error of the y-intercept and the slope of the linear regression (Table 2).

**Determination of Furosine by RP-HPLC.** Furosine formation was determined in milk after acid hydrolysis by RP-HPLC as described previously in ISO 18329:2004 (IDF 193:2004) with some modifications. One milliliter of milk was mixed with 3 mL of 10 M HCl in screw-cap tubes, and nitrogen was bubbled through the samples for 2 min. The tubes were closed and heated at 110−115 °C for 18 h. After heating, the samples were cooled and filtered through a filter paper (Black Ribbon 589/1, Schleicher & Schuell MicroScience GmbH, Dassel, Germany), and 2 mL of filtrate was filtered through a 0.45 μM disposable filter, diluted 5 times in 3 M HCl, and transferred into HPLC vials. Furosine detection was performed on a Dionex 300DX system (Dionex, Germering, Germany) with a DAD detector and using a Supelco Supercosil LC-8 column (Sigma-Aldrich Inc.) with isocratic elution with 0.06 M sodium acetate buffer at a flow rate of 1 mL/min. The injection volume was 10 μL, and the DAD detection (UV) was at 280 nm.

**Determination of Free Amino Acids Using 1H NMR Spectroscopy.** Determination of free amino acids was performed using 1H NMR spectroscopy analysis according to a method adapted from Sundekilde et al. The milk filtrates were thawed, and 400 μL was mixed with 200 μL of D2O containing 0.025% sodium trimethylsilyl-[2,2,3,3-2H4]-1-propionate (TSP) as internal chemical shift reference. A Bruker Avance III 600 spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5 mm TXI probe was used for the analyses. The sample sequence was randomized prior to acquisition. Standard one-dimensional spectra were acquired using a single 90° pulse experiment with a relaxation delay of 5 s. Water suppression was achieved by irradiating the water peak during the relaxation delay, and a total of 64 scans were collected into 32K data points scanning a spectral width of 12.15 ppm. All 1H spectra were initially referenced to the TSP signal at 0 ppm. Prior to Fourier transformation the data were multiplied by a 0.3 Hz line-broadening function. The proton NMR spectra were phase and baseline corrected manually using Topspin 3.2 (Bruker Biospin). Amino acid concentrations were calculated for valine, methionine, isoleucine, alanine, and acetate using Chenomx software (Chenomx NMR suite 7.7, Edmonton, Canada).

**Determination of Free Amino Terminals by Fluorescamine Assay.** The level of free amino terminals in the milk was determined according to the method of Dalsgaard et al. An amount of 75 μL of milk was mixed with 75 μL of 24% trichloroacetic acid (24 g TCA/100 mL water) and placed on ice for 30 min to accelerate protein precipitation. The precipitated mixture was centrifuged at 17049g for 20 min. A total of 37.5 μL of supernatant was then added to 1130 μL of 0.1 M borate buffer and 375 μL of fluorescamine solution (0.2 mg/mL fluorescamine in water free acetone), and 250 μL was then transferred.
to each well in a microtiter plate. The analysis was carried out, 18 min after the addition of the fluorescamine, on a Synergy 2 Multi-Mode microplate reader (Holm & Halby, Brøndby, Denmark) with excitation at 390 nm and emission at 480 nm. Quantification was achieved by calculating leucine equivalents using an external leucine standard curve prepared as follows. A 0.1 M leucine stock solution was made (327.5 mg of leucine in 25 mL of 1 mM HCl), and from the stock solution six different concentrations were made (0.0005–0.0030 M leucine) and mixed with 10 mL of 1 mM HCl and analyzed as the milk samples.

**Statistical Analysis.** To identify significant effects of milk type, storage time, and degree of increase in concentration on the quantified volatile compounds, furosine, and free amino acids, regressions were made by a linear model (lm) followed by a one-way analysis of variance (ANOVA). In addition, a linear mixed model (lmer), least-squares means (LSMEANS), and Tukey’s honest significant difference (α = 0.05) test was performed using the software R (version 3.0.2, R Foundation for Statistical Computing, Vienna, Austria).

### RESULTS

**GC-MS, NMR, furosine, and fluorescamine analyses** were conducted on industrially produced conventional UHT milk (100% lactose) and lactose-hydrolyzed UHT milk (<1% of original level of lactose) throughout a storage period of 9 months at 22 °C. The lactose-hydrolyzed UHT milk was heated with 60% of the original level lactose, which was subsequently hydrolyzed to glucose and galactose by the addition of lactase. The compounds detected in the milk by the different analytical approaches are summarized in Figure 2.

**Furosine.** The development of furosine levels in the milk after acid hydrolysis was found to be significantly affected by milk type. A significantly higher initial level of furosine was observed in CONVI milk (p < 0.001) (Figure 3) compared to LHD milk and LHI milk; however, after approximately 60 days, furosine levels started to increase in LHD milk and LHI milk, whereas in CONVI milk furosine levels were constant throughout the storage period. After 270 days of storage, the furosine content was about 2.5 times higher in lactose-hydrolyzed milk than in conventional milk.

**GC-MS.** A total of 27 volatile compounds were extracted from the headspace sampling and identified using GC-MS (Table 2), and of these 15 were quantified (Table 3). The remaining 12 identified compounds (Tables 2 and 3) were constant throughout the storage period for all milk types and were therefore not quantified.

Notably, the levels of the five ketones quantified were significantly higher in conventional UHT milk (p < 0.001) than in lactose-hydrolyzed milk in the initial phase of the storage period by a factor of 4–5 (Figure 3). All of the quantified ketones followed a similar trend during storage for all milk types, and a significant (p < 0.001) increase in the concentration of ketones was observed during storage (Figure 4). However, for 2-pentanone (p < 0.001), 2-heptanone (LHD, p = 0.016; LHI, p = 0.006), and 2-nonanone (LHD, p < 0.001; LHI, p < 0.001) the formation rate significantly increased at a higher extent for lactose-hydrolyzed milk compared with conventional milk (Figure 4A–C; Supporting Information). In addition, the formation rate of 2-undecanone increased significantly more in LHD than in CONVI (p = 0.029) and LHI (p = 0.023) milks. 2-Nonanone was the only ketone for which a difference between direct and indirect heat treatment could be observed during storage and a higher formation rate was seen in LHD (p = 0.0012) milk compared with LHI milk.

2-Octanone did not appear before 90 days in lactose-hydrolyzed milk, whereas it was observed already at day 14 in conventional milk. A higher formation rate in the level of 2-octanone was observed in CONVI (p = 0.0224) milk compared to LHD milk during storage (Figure 4D).

With the exception of 2-methylbutanal, no significant change in the concentration was observed for the quantified aldehydes during storage (Figure 5). Notably, the concentration of 2-methylbutanal was almost constant in CONVI milk during the 9 month storage period, whereas its concentration (Figure 5D; Supporting Information) increased tremendously after 150 days of storage in LHD (p < 0.001) and LHI (p < 0.001) milk. In addition, it was observed that the level of 2-methylbutanal significantly increased more in LHI (p < 0.001) milk than in LHD milk from day 200.

Heterocycles 2-methylfuran and 2-ethylfuran exhibited a pattern similar to that of the ketones, and a significant increase in their concentrations was observed for all of the milk types during storage (p < 0.001) (Figure 6A,B; Supporting Information). The increase in concentration of the two furans during the storage period was significantly higher in CONVI (p < 0.001) milk compared with the lactose-hydrolyzed milk, and the concentration of 2-ethylfuran increased more in LHI (p = 0.012) milk compared with LHD milk. A different pattern was observed for 2-pentylfuran (Figure 6C). Whereas a significant increase in the concentration of 2-pentylfuran was observed in CONVI (p < 0.001) milk during the storage period, the concentration remained almost constant in LHD and LHI milk.

Dimethyl trisulfide (DMTS) increased in LHD and LHI milk up to 50 days of storage, followed by a decrease to below the initial concentration. In CONVI milk a higher initial concentration of DMTS was observed than in LHD and LHI milks; however, the concentration decreased rapidly during storage, and a low and constant concentration of approximately 0.2 μg/kg milk was reached after approximately 50 days of storage (Figure 7). Dimethyl disulfide (DMDS) was constant throughout the storage period for all milk types (data not shown).

**Analysis of Amino Acids by NMR.** Four free amino acids, isoleucine, alanine, methionine, and valine (Figure 8), were identified and quantified by 1H NMR spectroscopy. The levels of alanine (LHD, p < 0.001; LHI, p < 0.001), isoleucine (LHD, p = 0.002; LHI, p < 0.001), and valine (LHD, p < 0.001; LHI, p < 0.001) increased in lactose-hydrolyzed milk, whereas they remained constant in the conventional milk throughout the
Methionine content was initially significantly higher in CONVI ($p = 0.0011$) compared to LHI milk (Figure 8C; Supporting Information). Throughout the storage period methionine levels in CONVI and LHD milk decreased to the level of LHI milk.

In addition to the four amino acids, acetate was also quantified by $^1$H NMR spectroscopy (Figure 9; Supporting Information). The level of acetate significantly increased in storage period (Figure 8A,B,D; Supporting Information).
lactose-hydrolyzed milk (LHD, \(p = 0.028\); LHI, \(p = 0.0336\)) during storage, whereas the increase in conventional milk during storage was not significant.

**Analysis of Free Amino Terminals by Fluorescamine Assay.** Slightly higher initial levels of free amino terminals and amino groups of lysine side chains were observed in the CONVI milk compared with lactose-hydrolyzed milk (Figure 10). However, the level of free amino terminals, expressed as leucine equivalents, significantly increased in the lactose-hydrolyzed milk (\(p < 0.001\)), whereas it remained rather constant in the conventional milk during storage (Figure 10; Supporting Information). After 270 days of storage, the level of free amino terminals was about 2 times higher in lactose-hydrolyzed milk than in conventional milk. No differences were observed between LHD and LHI milks.

### DISCUSSION

In the present study volatile compounds, Amadori products as determined by furosine levels, levels of free amino acids, and levels of amino terminals, and proteolysis were analyzed in lactose-hydrolyzed and conventional UHT milk to elucidate the detailed chemical changes during a 9 month storage period (Figure 2).

**Maillard Reaction Products.** An increase in the level of furosine was observed in all milk types during the 9 month storage, which demonstrates a continuous formation of its precursors, lactulosyllysine and fructosyllysine (Figures 2 and 3). During the first 60 days of storage lactose-hydrolyzed milk was characterized by a significantly lower furosine level than conventional milk. This was likely due to the removal of approximately 40% of lactose prior to the UHT treatment in the lactose-hydrolyzed milk, resulting in lower levels of carbohydrates during the UHT treatment and lower levels of Maillard reactions in the initial storage period. This indicates that furosine precursors were formed during the heat treatment. However, during storage, the formation rate was significantly higher in lactose-hydrolyzed milk compared to conventional milk. This is consistent with the fact that the monosaccharides polymerize more progressively than disaccharides. This finding reveals that despite lower carbohydrate levels, the Maillard reaction proceeds more rapidly and to a greater extent in lactose-hydrolyzed milk during storage. Previous studies have followed the development of furosine in lactose-hydrolyzed and conventional milk for up to 4 months and showed significant increases in furosine during storage. Messia et al. investigated the formation of furosine in lactose-hydrolyzed and conventional indirect UHT milk during 4 months of storage at 20 °C and found higher values of furosine in the former. The concentrations of furosine found in the lactose-hydrolyzed milk at production and after storage for 4 months were 234 and 562 mg/100 g protein, respectively, whereas the corresponding concentrations found in the conventional milk were 235 and 310 mg/100 g protein, respectively. These results are quantitatively consistent with the results in the present study for the same storage period, temperature, and milk type. However, this study shows that it is important to follow the development of furosine for longer than 4 months (Figure 3). Tossavainen and Kallionen investigated furosine content in three milk types: nonhydrolyzed, hydrolyzed, and lactose-reduced-hydrolyzed (similar to the lactose-hydrolyzed milk in...
The level of furosine in conventional and lactose-hydrolyzed UHT milk is very dependent on the storage period. This has implications for understanding storage-induced deterioration of milk quality.

A very similar formation pattern was observed for the furosine generation and the Maillard reaction product, 2-methylbutanal (Figure 5D). After initially being observed at a lower concentration in LHI and LHD, which is consistent with total carbohydrate content, accumulation of 2-methylbutanal was found to occur more rapidly and to a much greater extent in lactose-hydrolyzed milk. This observation is in good agreement with the current understanding of the Maillard-Strecker degradation pathway. The volatile 2-methylbutanal is a Strecker degradation aldehyde and is formed when isoleucine reacts with dicarbonyl compounds in the milk. The increased concentration of 2-methylbutanal in lactose-hydrolyzed compared to conventional UHT milk can be ascribed to the type of sugar (lactose, glucose, and galactose) available in the milk. Kato et al. studied protein polymerization and browning in a model system consisting of ovalbumin, glucose, and lactose and concluded that glucose accelerates the protein polymerization as well as the Amadori degradation. Kato et al. also concluded that the Amadori compounds formed from lactose were less prone to degradation into aldehyde compounds compared with the Amadori compounds from glucose. Intriguingly, in the present study it could be observed that the free amino acid and precursor for Strecker degradation, isoleucine, increased during storage in lactose-hydrolyzed milk, whereas it remained constant in conventional milk (Figure 8A). This finding is probably related to a higher level of proteolysis in lactase-treated milk and could be due to unwanted side activities of the enzyme preparation. Balagiannis et al. investigated the formation of 2-methylbutanal and observed that the sugar concentration, the Amadori rearrangement, the rate of dicarbonyl formation, and the amount of isoleucine were the limiting steps of its formation. The simultaneous increase of isoleucine, furosine, and 2-methylbutanal in lactose-hydrolyzed milk after approximately 100–150 days of storage indicates that the level of isoleucine and the Amadori rearrangement is important for the formation of 2-methylbutanal, which, in addition to the lower reactivity of lactose with proteins and the nondetectable formation of isoleucine, explains the absence of a formation of 2-methylbutanal in the conventional UHT milk.

Furfural is formed in the 3-deoxyosone pathway in the early Maillard reaction, which is favored by acidic pH. However, furfural may be a precursor of the formation of furan derivatives in the Maillard reaction. Berg observed that furfural was formed in milk in very small concentrations after heating and that the level of furfural increased with increasing heat load. In the present study, furfural was identified and quantified, and it was found that furfural remained almost constant at a concentration of 0.8–6 μg/kg (8.3–62.4 μmol/kg) throughout the storage period. This concentration is higher than the concentration detected by Berg, who reported 0.68 μmol furfural/kg in UHT milk. This difference is possibly due to differences in the UHT treatment or differences in the sensitivity of the applied GC-MS method. The results in the present study indicate that no formation of furfural is taking place during storage at room temperature, likely due to the nonpreferred pathway at normal milk pH.

Acetate, identified by NMR spectroscopy in the present study (Figure 9), is a major degradation product of sugars in the Maillard reaction. In neutral and basic pH, acetate is formed,
primarily from C2–C3 β-cleavage of the 1-deoxyosone isomerization product 1-deoxy-2,4-hexodiulose (2,4-pentadione). Acetate is a stable end product of this pathway and considered as a marker for the 2,3-enolization in the Maillard reaction. In addition, acetate can also be formed directly from sugar fragmentation. In the present study the level of acetate increased during storage for all of the milk types, and no significant difference was found between the milk types (Figure 9). The accumulation of acetate in the milk during storage indicates that a continuous formation is taking place throughout storage and that the degradation of the respective Amadori compound (fructosyllysine in lactose-hydrolyzed milk and lactulosyllysine in conventional milk) occurs throughout storage, leading to a continuous formation of the dicarbonyls, which are included in Strecker degradation in the Maillard reaction (Figure 2). The comparable increase of acetate in lactose-hydrolyzed and conventional milk indicates that the same level of Amadori compound degradation is present in both milk types. Kato et al. observed that Amadori compounds from lactose were more difficult to degrade compared with the Amadori compounds from glucose, which could explain the lower level of Strecker aldehydes such as 2-methylbutanal in conventional milk in the present study. However, contrary to Kato et al., the present study indicates that the 2,3-enolization from the Amadori compounds to 1-deoxyosone is similar in conventional and lactose-hydrolyzed UHT milk and that the hindrance for further degradation to aldehydes is taking place after this step in the Maillard reaction cascade (Figure 2).

2-Methylfuran and 2-ethylfuran increased significantly during storage in all milk types (Figure 6), but a larger increase was seen in CONVI milk compared with LHD and LHI milks. The furans 2-methylfuran and 2-ethylfuran are Strecker aldehyde (acetaldehyde and lactaldehyde) products as well as sugar and protein degradation products. The higher content of furans in the conventional milk indicates that the formation of furans is favored by lactose, and not glucose and galactose. This suggestion is supported by a recent published work by Owczarek-Fendor et al., who investigated furan formation in starch-based systems containing various carbohydrates and proteins, heated at 130 °C for 30 min, at different pH values. The study revealed that lactose and lactose–protein systems
generated higher amounts of furan at pH 6 compared with glucose and fructose systems.

Thermal Degradation and Oxidation Products. In addition to 2-methylbutanal, four other aldehydes were quantified. The nonsystematic variation in aldehydes could potentially be ascribed to the low concentration of these compounds. During thermal processing fatty acids may undergo thermal degradation reactions, including isomerization, cyclization, and cleavage, and aldehydes are mainly formed by oxidation of fatty acids.29,30 Straight-chain aldehydes are the most important markers for fat oxidation.31 Vazquez-Landaverde et al. observed that aldehydes were not affected by heat to the same extent as ketones.2 Valero et al. found a small increase in aldehydes during 120 days of storage.7 Jeon et al. investigated the effect of addition of ascorbic acid, acting as antioxidant, on the formation of aldehydes in UHT milk and found that the addition of ascorbic acid reduced the formation of aldehydes.32 Osada and Shibamoto investigated the antioxidative activity of volatile extracts from Maillard model systems and observed that the combined effect of many compounds produced in the Maillard reaction possessed an antioxidative effect that decreased the oxidation of hexanal.33 Thus, in the present study, an antioxidative activity from the advanced Maillard reaction products34 or peptides and proteins35 formed by proteolysis, may inhibit the formation of aldehydes. This could explain the low level of change observed for the aldehydes during storage in all milk types. However, the low level of aldehydes may also indicate a low level of oxidation in the milk.

During storage a significant increase in the concentration of ketones was observed in the milks (Figure 4). Ketones are derived from fatty acids through β-oxidation36,37 and are primarily formed by thermal degradation.29 The ketones are known to be temperature-dependent, and increased levels of ketones have been observed in milk exposed to higher temperatures.2 The present study revealed that both the level and the formation rate of ketones were affected by milk type. Whereas the level of ketones was higher in conventional UHT in the beginning of the storage period, the levels of 2-pentanone, 2-heptanone, and 2-nonanone increased significantly in the lactose-hydrolyzed compared to conventional milk. In addition, a higher increase in 2-undecanone was observed in LHD milk compared with CONVI milk. These findings indicate that the thermal degradation of the fatty acids is more pronounced in lactose-hydrolyzed milk than conventional UHT milk. However, because the heat treatments applied were similar, the exact mechanism remains unknown.

A significant increase in 2-pentylfuran was found in CONVI milk during storage, whereas this compound was found to be constant in lactose-hydrolyzed milk (Figure 6C). Overall, 2-pentylfuran is formed from oxidation of unsaturated lipids.27,37 A possible explanation for the increased formation of 2-pentylfuran in the conventional UHT milk compared with the lactose-hydrolyzed milk may be a higher antioxidative activity in the lactose-hydrolyzed milk due to an increased Maillard reaction34,38 or increased proteolytic activity35 forming antioxidative compounds. The antioxidative compounds may decrease the oxidation of the unsaturated lipids present in the milk and thereby the furan formation.

When β-lactoglobulin is heat-denatured, methionine and cysteine are exposed to further reactions, such as oxidation and Maillard reactions, and Strecker degradation. The Strecker degradation of methionine may lead to the formation of methional, which further oxidizes to DMDS and DMTS,36,39 and in the present study, DMTS apparently was formed after or during heat treatment in all milk types. In lactose-hydrolyzed milk the DMTS concentration increased further during storage and then decreased rapidly after approximately 50 days (Figure 7), whereas an immediate decrease was observed in the conventional UHT milk. In addition, it was observed that methionine decreased in lactose-hydrolyzed milk, which could indicate that methionine was degraded to DMTS in the initial part of the storage period. However, for the conventional UHT milk, methionine first increased followed by a slow decrease, and the decrease could not be correlated to any simultaneous formation of DMTS. Sulfides and sulfur-containing amino acids are very reactive, and the decrease of these compounds during storage is mainly thought to be due to degradations and oxidations.40,41 Because of observations of increased levels of free amino acids or peptides in lactose-hydrolyzed milk during storage (Figure 8A,C,D), a similar observation was expected with methionine; however, no increase was observed. This may be explained by the specificity of the proteolytic enzyme, which may not cleave methionine or methionine-containing peptides, or rapid degradation of exposed methionine residues, which would prevent its detection in the NMR.

Protein Hydrolysis during Storage. In the present study 1H NMR spectroscopy was used to determine amino acids acting as precursors in Maillard reaction and also to determine acetic acid, which can be formed during the Maillard reaction. In addition to methionine and isoleucine already discussed, alanine and valine were quantified in the milk by 1H NMR spectroscopy. This quantification revealed that these amino acids increased significantly in lactose-hydrolyzed milk during storage, whereas they remained constant at a lower level in the conventional milk (Figure 8B,D). In addition, an increase in the level of free amino terminals, obtained from the fluorescamine assay, was observed in lactose-hydrolyzed milk compared to conventional UHT milk in Figure 10. These results demonstrate a higher proteolytic activity in lactose-hydrolyzed milk compared to conventional milk. An increased level of free amino terminals and amino groups of lysine side chains in lactose-hydrolyzed milk may contribute considerably to the higher level of Maillard reaction products in the lactose-hydrolyzed milk. An increased Maillard reaction decreases lysine availability in lactose-hydrolyzed milk, and therefore the protein nutrition value may be lower in this type of milk. A lower protein availability might pose a challenge when aiming at decreased malnutrition in countries with a high prevalence of lactose intolerance. Thus, it is of interest to optimize the production of lactose-hydrolyzed milk to avoid high levels of Maillard reactions and thereby maintain a high nutritional value of the milk. The increased level of free amino acids and amino terminals (Figures 8 and 9) in lactose-hydrolyzed milk during storage indicates that the proteolytic activity is more pronounced than the Maillard reaction activity. Tossavainen and Kallioinen analyzed proteolytic activity in lactose-hydrolyzed and unhydrolyzed UHT milk directly after production and after 4 weeks of storage and observed a higher proteolytic activity in the hydrolyzed UHT milk compared to the unhydrolyzed UHT milk, which is in agreement with the present study.10 The higher proteolytic activity is probably due to the proteolytic side effect of the added lactase enzyme preparation, which gives rise to substrates for the Maillard reaction and thereby an increased risk for milk quality deterioration during storage.
Effect of Heat Treatment. In addition to the effect of lactose hydrolysis, the effects of heat treatment were also elucidated in the present study as two different types of heat treatments were included: a direct and an indirect heat treatment. However, no differences in detection in furosine content or free amino terminals were observed between direct and indirect heat-treated lactose-hydrolyzed milks. Higher furosine levels in conventional indirect UHT milk compared with conventional direct UHT milk have been reported in previous studies. The levels of 2-nonenone and 2-undecanone increased more in LHD than in LHI milk, which is contrary to the observation of Perkins et al., who found an increased total amount of ketones in indirect heat-treated conventional UHT milk, compared with the direct heat-treated milk. The significantly higher increases in 2-methylbutanal and 2-ethylfuran during storage in LHI compared with LHD milk could be explained by the different heat loads from direct and indirect heat treatments. To the best of our knowledge, no studies comparing direct and indirect heat treatments of lactose-hydrolyzed milk, where lactose is removed and hydrolyzed after heat treatment, have been reported.

The present study, which included several complementary analyses, shows that the type of carbohydrate has a greater impact on the Maillard reaction, heat load indicators such as furosine, and total concentration of ketones, compared to the impact of different heat treatments (direct and indirect UHT). The formation of 2-methylbutanal and furol was considerably higher in lactose-hydrolyzed milk than in indirect UHT milk. In addition, an increase in free amino acids and free amines increased tremendously in lactose-hydrolyzed milk compared with conventional UHT milk after a 9 month storage period. Residual proteolytic activity from the lactase-enzyme preparation may be responsible for the higher levels of free amino acids observed in lactose-hydrolyzed UHT milk. This study reveals that lactose-hydrolyzed UHT milk, compared to conventional UHT milk, is more vulnerable to reactions that can impair the milk quality, primarily the Maillard reaction and proteolysis but, to some extent, also oxidation.

ASSOCIATED CONTENT

Supporting Information
Overview of storage and effects on the amount of volatile compounds in lactose-hydrolyzed direct UHT milk (LHD), lactose-hydrolyzed indirect UHT milk (LHI), and conventional indirect UHT milk (CONVI). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED
CONVI, conventional indirect UHT milk; DMDS, dimethyl disulfide; DMTS, dimethyl trisulfide; DSQ, dual-stage quadrupole; ESL, extended shelf life; HCl, hydrochloric acid; HPLC, high-pressure liquid chromatography; GC-MS, gas chromatography–mass spectrometry; LHD, lactose-hydrolyzed direct UHT milk; LHI, lactose-hydrolyzed indirect UHT milk; LME, linear mixed model; LSMEANS, least-squares means; LOD, limit of detection; LOQ, limit of quantification; NMR, nuclear magnetic resonance; TIC, total ion current; SIM, selected-ion monitoring; UHT, ultra-high temperature; UV, ultraviolet.

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Paper 3

Chemical and proteolysis-derived changes during long-term storage of lactose-hydrolyzed UHT milk


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Chemical and proteolysis-derived changes during long-term storage of lactose-hydrolyzed UHT milk

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Abstract

Proteolytic activity in milk may release bitter-tasting peptides and generate free amino terminals that react with carbohydrates which initiate the Maillard reaction. Ultra high temperature (UHT) heat treatment inactivates the majority of proteolytic enzymes in milk, however, in lactose-hydrolyzed milk a β-galactosidase preparation is applied to the milk after heat treatment. This enzyme preparation has proteolytic side-activities that may induce quality deterioration of long-term stored milk. In the present study proteolysis, glycation and volatile compounds formation were investigated in conventional (100% lactose), filtered (60% lactose) and lactose-hydrolyzed (<1% lactose) UHT milk using reverse phase-high pressure liquid chromatography-mass spectrometry, proton nuclear magnetic resonance, and gas chromatography-mass spectrometry. Proteolysis was observed in all milk types, however, the degree of proteolysis was significantly higher in the lactose-hydrolyzed milk compared to the conventional and filtered milk. The proteins most prone to proteolysis were β-CN and αs1-CN, which was clearly hydrolyzed after approximately 90 days of storage in the lactose-hydrolyzed milk.

Keywords: Lactose-free milk, proteolysis, heat treatment, lactose hydrolysis, Maillard reaction, Shelf-life
Introduction

During storage of ultra-high temperature (UHT) heated milk, the Maillard reaction, oxidation and proteolysis proceeds and changes the chemical composition of the milk. It has been shown that lactose-hydrolyzed milk manufacturing favors initiation of the Maillard reaction compared to conventional UHT milk.\textsuperscript{1–3} This fact can be ascribed to a higher reactivity of glucose and galactose with the proteins in the milk (mainly lysine) as compared with lactose.\textsuperscript{4,5} Moreover, a higher proteolytic activity has been observed in lactose-hydrolyzed milk,\textsuperscript{6,7} which through the formation of peptides and amino acids likewise favors Maillard reactions and add to flavor-active compounds and thereby contribute to the formation of a stale aroma and flavor.\textsuperscript{8–11} Heat treatment decreases the proteolysis in milk due to denaturation of the proteolytic enzymes. However, during long-term storage, proteolytic activity may deteriorate the milk even in products that have been exposed to a UHT treatment.\textsuperscript{12} The activity of plasmin may cause bitterness, gelation and precipitation during long-term storage which decreases the shelf-life of UHT milk.\textsuperscript{13} In lactose-hydrolyzed milk β-galactosidase is added before or after the heat treatment of the milk to hydrolyze the lactose. The β-galactosidase enzyme preparations may contain undesired proteolytic side-activities especially if the enzyme is added after heat processing of the milk, since the enzymes are not inactivated and the milk is stored for several months at room temperature. Few studies have investigated the proteolytic activity in lactose-hydrolyzed UHT and only for up to 3 months of storage.\textsuperscript{6,7,14,15} Consequently, a detailed understanding of the chemical changes in lactose-hydrolyzed milk during long-term storage is lacking. The aim of the present study was to get a further understanding of the chemical changes taking place in enzymatically lactose-hydrolyzed UHT milk and conventional UHT milk during a 9-months storage period. This was achieved through an elucidation of the concomitant proteolysis, glycation and volatile compounds formation by using a combination of reverse liquid chromatography-mass spectrometry (RP-HPLC MS), proton nuclear magnetic resonance ($^1$H NMR) spectroscopy, and gas chromatography-mass spectrometry (GCMS).
Material and methods

Chemical references

Bis-Tris, D$_2$O containing 0.025% sodium trimethylsilyl-[2,2,3,3-$^2$H$_4$]-1-propionate (TSP), 1,4-dithioerythritol, Guanidine hydrochloride, Sodium-citrate dehydrate, Trifluoroacetic acid were obtained from Sigma-Aldrich Inc. (Steinheim, Germany). Acetonitrile was purchased from Mikrolab RA 1016 (Rathburn Chemicals, Ltd. Walkernburn, UK)

Milk samples

Four types of milk (conventional indirect UHT, lactose-hydrolyzed direct UHT, lactose-hydrolyzed indirect UHT, and filtered (lactose-reduced) indirect UHT) were produced at a commercial Danish dairy plant (Arla Foods, Esbjerg, Denmark) (Table 1). All the milk were UHT heated using indirect heat treatment where a tubular heat exchanger was used to heat the milk, followed by slow cooling (140-145 °C for 2-3 sec.). An additional lactose-hydrolyzed milk batch was heat-treated using direct heat treatment where steam was injected into the milk, followed by flash cooling (140-145 °C for 2-3 sec.). Ultra and nano-filtration were applied to reduce the lactose content to approximately 60% in the lactose-hydrolyzed and the filtered milk before heat treatment. The remaining lactose was enzymatically hydrolyzed after heat treatment, by addition of β-galactosidase (Maxilact LAG 5000, DSM, Netherlands). All milk samples were packaged aseptically in 1 Liter Tetra Brik® cartons.

The milk samples were stored in an air-conditioned room at 22 °C. After 14, 21, 28, 45, 60, 90, 120, 150, 180, 210, 240, 270 days of storage one carton of each milk type was opened for analyses. Milk samples for $^1$H NMR spectroscopic analysis were filtered using Amicon Ultra 0.5 mL 10 kDa (Millipore, MA) spin filters at 10,000 g for 15 min and subsequently frozen at -80 °C in 2 mL tubes. Milk samples for RP-HPLC MS analysis were skimmed by centrifugation (Sorvall® RC 5B plus,
Thermo, Waltham, Massachusetts, US) at 12800 g for 30 minutes, 4 °C, and subsequently frozen at
-80 °C in 2 mL tubes.

**Aroma profiling by dynamic headspace sampling and GCMS**

Dynamic headspace sampling and gas chromatography-mass spectrometry (DHS GCMS) was used
to extract and analyze the volatile compounds in the fresh, unfrozen milk according to the procedure
described by Jansson et al. 2014. Adsorbent traps (Tenax TA, 200 g Tenax TA, 35/60 mesh
(Markes International Limited, Llantrisant, UK)) were used to isolate the volatile compounds which
were then desorbed (250 °C, 15 min) onto a cold trap (U-TIGCP, Markes International Limited)
using a thermal desorption system (ultra-UNITY™, Markes International Limited). Subsequently,
the volatiles were injected into a gas chromatograph (Finnigan trace GC ultra, Thermo, Waltham,
Massachusetts, US) and separated using a CP-Wax 52CB column (50 m x 0.25 mm, film thickness
0.25 µm, (Varian, Palo Alto, CA)). The separated volatile compounds were detected using a single
quadrupole mass spectrometer (Finnigan Trace dual-stage quadrupole (DSQ), Thermo). The mass
spectrometer scanned over a mass to charge (m/z) range of 45-650 (4.46 scans/sec) in TIC mode. In
total 28 compounds were identified using the NIST mass spectral library (NIST MS search 2.0,
2011) and external standards. Four of these: dimethyldisulfide (DMDS), dimethyltrisulfide
(DMTS), 2-ethylfuran, 2-pentylfuran) were quantified using external standard curves as previously
described.

**Metabolite profiling by 1H NMR spectroscopy**

Prior to proton nuclear magnetic resonance (1H NMR) spectroscopy, the milk filtrates were thawed
in randomized order and analyzed as described previously by Jansson et al 2014: Four hundred µL
milk was mixed with 200 µL D2O containing 0.025% TSP as an internal standard. The samples
were analyzed on a Bruker Avance III 600 spectrometer (Bruker Biospin, Germany) equipped with
a 5 mm TXI probe. From a single 90° pulse experiment with a relaxation delay of 5 s standard one-
dimensional spectra were obtained. The water peak was suppressed by irradiation during the
relaxation delay, and a total of 64 scans were collected into 32K data points scanning with a spectral
width of 12.15 ppm. The data were multiplied by a 0.3 Hz line-broadening function and
subsequently Fourier transformed. The TSP signal at 0 ppm was used as a reference, and all the $^1$H
spectra were manually baseline and phase corrected using Topspin 3.2™ (Bruker Biospin). The
sugar regions at 5.29-5.22, 5.0-4.49 and 4.11-3.32 ppm and the region with citrate at 2.74-2.47 ppm
were removed from the data to facilitate the interpretation of the other metabolites. Metabolite
concentrations were calculated for alanine, isoleucine, leucine, lysine, methionine, tryptophan,
tyrosine, valine, formate lactose, glucose and galactose, using Chenomx NMR suite 7.7 (Chenomx,
Edmonton, Canada).

**Protein profiling and determination of glycation by RP-HPLC MS**

The RP-HPLC MS procedure used in the present study was modified according to Jensen et al.
(2012) and the procedures for sample preparation, settings for the HPLC, ESI source, and the
mass selective detector were as described by Frederiksen et al. (2011). Proteins were separated by
reversed phase chromatography using an Agilent 1100 system (Agilent Technologies, Santa Clara,
CA) with a Jupiter C4 column (250 mm x 2 mm, 5µM particle size, 300 Å pores; Phenomenex,
Torrance, CA) operated at 40 °C and a G1315A diode array detector with Ultra Violet (UV)
detection at 214 nm coupled to a mass selective detector for identification and relative
quantification. Average molecular masses (Mw) of the milk proteins were obtained using the
deconvolution algorithm of the ChemStation software (rev.B.04.01 SP [650], Agilent
Technologies). All milk samples were analyzed in technical duplicates. The relative protein content
of the intact milk proteins was calculated as the integrated area of each intact protein normalized
with total integrated peak area in each LC-chromatogram. ‘Total CN’ comprised the sum of $\alpha_{S1}$-
CN, $\alpha_{S2}$-CN, $\beta$-CN, and $\kappa$-CN.
Statistical analysis

Prior to multivariate data analysis, \(^1\)H NMR spectroscopic data were mean-centered and Pareto-scaled. Principal component analysis (PCA) was applied to the centered and scaled data to elucidate differences between the milk types. A partial least square (PLS) regression model was used to predict the storage time, and a corresponding variable important in projection (VIP) plot was applied to identify the metabolites correlating strongest with the storage-induced changes. The PLS model was cross-validated (CV) using segmented CV with nine splits, where it was ensured that each sample replicate was in the same split. The root mean square error of cross validation (RMSECV) was used as a measure of model performance. PCA was performed using SIMCA P+ 13.0 (Umetrics, Umeå, Sweden) and PLS was performed using PLS Toolbox 6.71 (Eigenvector Research Inc. Wenatchee, WA) in MATLAB 7.11 (Mathworks, Natick, MA).

To test the effect of milk type and storage time on the quantified proteins, amino acids, formate, carbohydrates and the Strecker degradation compounds linear mixed models (lme), least square means (lsmeans), and Tukeys honest significant differences test (\(\alpha = 0.05\)) were calculated in R (version 3.0.2, R Foundation for statistical Computing, Vienna, Austria).
Results

Chemical changes in UHT-heated milk stored at 22 °C were followed over a period of nine months. H NMR spectroscopy was applied to follow proteolysis through the detection of free amino acids. In total, nine free amino acids could be quantified (Table 2) and most of them increased significantly in the lactose-hydrolyzed milk during storage (Figure 1), while they remained constant or were not detected (phenylalanine, tryptophan and tyrosine) in the filtered and conventional UHT milk (Figure 1). Only exceptions were methionine which was constant throughout the storage period and lysine which increased in all milk types.

In order to investigate which proteins that gave rise to the increased level of amino acids in the lactose-hydrolyzed UHT milk, the intact proteins in the milk were analyzed using RP-HPLC MS. Proteolysis was observed for all proteins in all milk types during storage. However, the level of proteolysis was significantly higher in the lactose-hydrolyzed UHT milk compared to the filtered and conventional UHT milk (Table 2). The relative amount of total intact CN was reduced by 56% for lactose-hydrolyzed indirect UHT-heated milk and by 61% for lactose-hydrolyzed direct UHT-heated milk during the storage period, as compared to 15% for conventional and filtered indirect UHT-heated milk (Table 2). The two most extensively hydrolyzed proteins during storage were β-CN and α_{S1}-CN (Figure 2), which were reduced by approximately 80% and 45%, respectively (Table 2), in lactose-hydrolyzed milk compared to a more moderate reduction for conventional and filtered milk. κ-CN and β-LG decreased in all milk types, (Table 2), while α_{s2}-CN only decreased in the filtered and lactose-hydrolyzed UHT milk.

The identified amino acids and their involvement in Strecker degradation product formation is summarized in Table 3. Five putative Strecker degradation products were identified (Table 2) however no clear connection between the volatile compounds and the increased levels of free amino
acids could be observed. Likewise no clear effects of lactose hydrolysis, filtration or heat treatment on volatile compound formation were revealed (Table 2).

Using RP-HPLC MS we detected glycated proteins in all milk samples at all storage times as N-linked glycans with mass addition of 162 Da for one hexose and 324 Da for lactose, or multiple hexoses. Furthermore hydrophilic glycoproteins were identified as the first peak eluting from the RP-HPLC column. This peak increased significantly during storage in all milk samples, and this increase was highest in indirect heated lactose-hydrolyzed milk (Table 2). Even though protein glycation was observed, the level of free carbohydrates remained constant throughout the storage time. Though, after 240 days of storage a decrease in glucose and galactose content was observed in all lactose-hydrolyzed milk samples (data not shown), which is consistent with a decrease in the concentration of free amino acids, as shown in Figure 1.

Finally, formate can be formed from the deoxyosones and may therefore be an indicator of the Maillard reaction. It was found that initial formate concentrations were highest in conventional milk, however during storage similar formation rates were observed for all milk types, except for the filtered milk (Table 2).

Further, the $^1$H NMR spectroscopic data were analyzed using an untargeted approach in order to elucidate how storage affected overall metabolite composition. PCA of $^1$H NMR spectroscopic data showed a separation along PC1 and PC2 between the filtered (lactose-hydrolyzed and filtered UHT milk) and non-filtered (conventional UHT milk) samples, probably due to the dilution occurring in this step. In fresh samples (< 50 days old), no clear separation between lactose-hydrolyzed milk and filtered milk could be observed. In addition, along PC1 a pronounced effect of storage was observed for lactose-hydrolyzed milk, while for the conventional and filtered milk the storage effect was much smaller. A PLS regression model with $^1$H NMR spectroscopic variables as X and storage time as Y, was generated for each milk type (Figure 4A, 4C, 4E). The models revealed that the storage
time of lactose-hydrolyzed and filtered milk could be predicted within a variance of 11 days ($R^2$ CV was 0.99 and 0.98 respectively; RMSECV was 11.23 days and 11.45 days respectively), and the storage time of conventional milk could be predicted within a variance of 19 days ($R^2$ CV was 0.95 and RMSECV was 18.59 days). In order to find the metabolites responsible for the changes during storage VIP scores were plotted for each PLS model (Figure 4B, 4D, 4F). Choline was significant in all milk types, whereas amino acids (leucine, isoleucine, valine, alanine, phenylalanine), as expected, were much more important in lactose-hydrolyzed milk as compared to the other milk types. In the non-hydrolyzed milk samples hippurate, creatine/creatinine, N-acetyl carbohydrates and acetate were identified as significant.
Discussion

Storage stability is of importance in UHT milk to maintain the consumer acceptability of the milk. In the present study, chemical changes that could be detrimental to milk quality were investigated in UHT milk produced with and without lactose hydrolysis during a storage period of nine months. Furthermore, filtered indirectly heated UHT milk was included to follow the effect of the β-D-galactosidase added to the milk. Two different heat treatments (direct and indirect) applied to the lactose-hydrolyzed milk were included to investigate the effect of severity of heat treatment. However, the type of heat treatment did not have any effect on volatile compounds, metabolite profile or the proteolytic activity, and therefore the term lactose-hydrolyzed milk refers to both milk types.

Proteolysis has major effect in lactose-hydrolyzed milk

$^1$H NMR spectroscopy was used to identify metabolites in the milk, and PCA of $^1$H NMR spectroscopic data revealed much more severe storage-induced changes in lactose-hydrolyzed milk than in the conventional and filtered UHT milk (Figure 3). This result shows that major chemical changes are not induced by the filtration but rather they can be attributed to the addition of the β-D-galactosidase preparation. The PLS models calculated (Figure 4) could predict the time of storage of each milk with a high precision, as reflected in the low RMSECV values (11-19 days). However, the prediction was considerably better for lactose-hydrolyzed UHT milk than conventional UHT milk which supports the more severe storage-induced changes in the lactose-hydrolyzed UHT milk seen in the PCA. It is highly relevant to investigate these changes in more detail to obtain an increased understanding of the underlying mechanisms. In the present study proteolysis was the major chemical change observed. Interestingly lysine was the only amino acid that increased in all milk types during storage and this could be due to the presence of heat stable endogenous proteases which cleave proteins after lysine and arginine (Lys or Arg in $^1$ position). A decrease in lysine could be expected due to its involvement in the Maillard reaction. However, according to the results
in the present study the binding of carbohydrates to lysine seems to be of minor importance compared to the proteolytic activity.

The increased proteolytic activity was further elucidated by RP-HPLC MS by investigating the content of intact protein during storage. Our study showed that the caseins present in the lactose-hydrolyzed UHT milk were considerably affected by the proteolytic activity, while in the conventional UHT milk only a moderate proteolytic activity could be observed during storage. This is in agreement with the results from Tossavainen and Kallioinen (2007) who investigated lactose-hydrolyzed and conventional UHT milk stored at different temperatures (5, 22, 30 and 45°C) for 12 weeks. In addition, Tossavainen and Kallioinen (2007) found that the αs1, αs2, and β-CN were the most extensively degraded proteins, while β-LG, α-LA and κ-casein were less affected. The proteolytic activity in the filtered milk did not deviate from the conventional UHT milk, which indicates that no proteolysis originated from the filtration step. The most hydrolyzed proteins in the lactose-hydrolyzed UHT milk were β-CN and αs1-CN, which significantly decreased after 90 days of storage, while only a minor decrease was observed in the conventional and filtered UHT milk (Table 2, Figure 2). This is an important and interesting pattern depicting differences between the lactose-hydrolyzed and conventional UHT milk during storage. Another interesting observation was that the free amino acids increased immediately during storage while the reduction in β-CN and αs1-CN was not observed before after 90 days in the lactose-hydrolyzed milk. These findings could indicate that a low degree of hydrolysis of proteins gives rise to an increase in the level of free amino acids but do not affect the proteins sufficiently to be detectable by RP HPLC MS.

The decrease in β-CN and αs1-CN after approximately 90 days of storage (Figure 2) seems to relate with the development of an off-flavor, which typically develops after 90 days. Casein contains a high amount of hydrophobic amino acids, which are highly correlated to bitterness, and a proteolysis of casein and especially β-CN and αs1-CN would consequently result in a bitter taste. McKellar et al. (1984) investigated proteolysis and bitterness in UHT milk during 8 months.
storage at ambient temperature and observed a high correlation between storage time, proteolysis, and bitterness. In addition, McKellar et al. (1984) found a higher level of proteolysis and an earlier formation of bitter flavor in direct heated UHT milk compared to the indirect heated UHT milk.

The Maillard reaction in the lactose-hydrolyzed milk

The Maillard reaction is dependent on the reaction between amines (primarily protein-bound lysine and N-terminals) and reducing carbohydrates, and the type and concentration of these substrates are highly important for the level of Maillard reaction product formation. In the present study protein glycation, formate and Strecker degradation product formation were investigated in filtered, lactose-hydrolyzed and conventional UHT milk. The relative content of the glycoprotein increased during storage for all milk types (Table 2), although the absolute degree of glycation could not be quantified based on the RP-HPLC MS method applied. This is in contrast to the study by Carulli et al. (2011) who observed a higher degree of glycation of α-LA and β-LG in presence of glucose and galactose compared to lactose. Likewise, we were not able to detect a significant difference in the degree of formate or in the Strecker degradation product formation analyzed (2-pentylfuran, 2-methylfuran, DMDS, DMTS and benzaldehyde) during storage.

Strecker aldehydes have been shown to affect the milk aroma and both lactose hydrolysis and proteolysis were expected to promote the formation of Strecker aldehydes in lactose-hydrolyzed milk as compared to conventional UHT milk. However the lack of significant differences could indicate that the Maillard reaction may not the most important pathway for their formation. For instance benzaldehyde was observed in all milk types, while its precursor, phenylalanine, was only detected in lactose-hydrolyzed milk (Table 2). Valero et al. (2001) observed an increase of benzaldehyde in skim milk during storage. However, the present study could not support this observation.
We previously observed a striking correlation between 2-methylbutanal, isoleucine and furosine formation in lactose-hydrolyzed UHT milk after approximately 90 days of storage.\(^1\) This seems to be closely connected to the decrease in intact casein concentrations observed here. Hence, a possible link between proteolysis, glycation and Strecker aldehyde formation seems likely, though we cannot prove causality.

A final aspect that might affect the quality of lactose-hydrolyzed milk is the dilution that occurs during the filtration step. Due to the filtration lactose-hydrolyzed and filtered UHT milk were heat-treated with a 40% lower level of lactose compared to the conventional milk. This was reflected by a lower formate level in fresh lactose-hydrolyzed and filtered milk (Table 2), but did not result in different rates of formate formation during storage. The most significant pathway for formate generation is through \(\alpha\)-dicarbonyls generated in the Maillard reaction\(^{36,37}\) but our results indicate that the type of sugar as well as proteolysis are not decisive factors for the formation of formate in milk.

The present study shows that the side-effects of the added \(\beta\)-galactosidase are highly relevant for the chemical reactions taking place in the lactose-hydrolyzed milk during storage. Consequently, an alternative enzyme preparation method would be attractive for the production of lactose-hydrolyzed milk in order to decrease the observed problems. A possible alternative could be to add the enzyme before the heat treatment to inactivate all the enzymes. Tossavainen and Kallioinen (2007)\(^6\) investigated how heat treatment before and after addition of \(\beta\)-galactosidase to lactose-hydrolyzed milk affects the proteolytic activity, but no significant difference was observed between the different enzymatic treatments. In milk hydrolyzed prior to the heat treatment, a higher concentration of \(\beta\)-galactosidase is added compared to the milk hydrolyzed after the heat treatment to obtain the same hydrolytic effect. However, the implication of this is that a higher amount of
enzyme is active after heat treatment. Another alternative, and possibly a more suitable method would be to avoid the side-activity from the enzyme by applying a more pure enzyme preparation to the milk, and thereby decreasing the proteolysis in lactose-hydrolyzed milk. This calls for further investigations into the commercial β-galactosidase enzymes used during production of lactose-hydrolyzed products.

The generation of free amino acids during storage of lactose-hydrolyzed milk originates most likely from either enzymatic impurity in the used β-galactosidase preparation and/or side-activities of the β-galactosidase. Higher levels of proteolytic activity contribute to the flavor of the milk through formation of flavor-active amino acids/peptides and/or through formation of additional Maillard reaction products, e.g. Strecker aldehydes. The reactivity of glucose and galactose determines the rate of the Maillard reaction, however, in addition, our results indicate that a sufficient level of free amino acids is required in order to accelerate the Maillard reaction. In order to gain an improved understanding of the chemical changes observed in lactose-hydrolyzed milk it would be relevant to focus on the proteins β-CN and αs1-CN, which are most affected by the proteolytic activity, and analysis of the peptide profile of the hydrolyzed proteins would be relevant to identify the enzymes responsible for the proteolysis. In addition, investigations on the enzyme purity and how it affects the chemical changes observed in the lactose-hydrolyzed UHT milk would probably facilitate the development of lactose-hydrolyzed products with a long shelf-life.

**Abbreviations**

CN, Casein; CV, cross-validated; Da, Dalton; DMDS, Dimethyl disulfide; DMTS, Dimethyl trisulfide; DSQ, Dual-stage quadrupole; RP HPLC, Reversed phase high pressure-liquid chromatography; GCMS, Gas chromatography-mass spectrometry; LA, Lactalbumin; LG, Lactoglobulin; LME, Linear mixed model; LSMEANS, Least square means; MS, Mass
spectrometry; NMR, Nuclear magnetic resonance; PC, Principal component; PCA, Principal component analysis; PLS, Partial least square; RMSECV, Root mean square error of cross validation; TIC, Total ion current; TSP, sodium trimethylsilyl-[2,2,3,3-$^2$H$_4$]-1-propionate; UHT, Ultra-high temperature; UV, Ultraviolet; VIP, Variable importance in projection;
Acknowledgment

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*Food Res. Int.* **2014**. Submitted.


Figure captions

**Figure 1.** Concentration of isoleucine in conventional, lactose-hydrolyzed and filtered UHT milk, quantified by $^1$H NMR spectroscopy.

**Figure 2.** Relative quantification of $\alpha_s$1-casein and $\beta$-casein in conventional, lactose-hydrolyzed and filtered UHT milk as function of storage.

**Figure 3.** PCA score plot of $^1$H NMR spectroscopic data of conventional, filtered and lactose-hydrolyzed UHT milk during storage. Samples are colored according to days of storage. The first principal component explains 43.2% of the variation and the second principal component explains 20.1% of the variation.

**Figure 4.** PLS regression plots from $^1$H NMR data spectroscopic with predicted versus actual and corresponding VIP plot for model based on (A, B) conventional UHT milk (n=33), $R^2$ CV: 0.95, RMSECV: 18.59 days, (C, D) lactose-hydrolyzed UHT milk (n=65), $R^2$ CV: 0.98, RMSECV: 11.23 days, and (E, F) filtered UHT milk (n=23), $R^2$ CV: 0.98, RMSECV: 11.45 days. The numbers indicate the metabolites important for the regression; 1, 2, 3) Leucine, isoleucine, valine, 4) Alanine, 5, 6) Lysine, Leucine, 7) Acetate, 8) N-acetyl carbohydrates, 9, 10) Methionine, 11) Not identified 12, 13) Creatine/creatinine, choline, 14, 15, 16, 17) Hippurate, tyrosine, tryptophan, phenylalanine.
Tables

Table 1. Overview of milk types, heat treatment and lactose level included in the study. All milk contained 1.5% w/w fat and 3.5% w/w protein.

<table>
<thead>
<tr>
<th>Milk</th>
<th>Enzymatic hydrolysis</th>
<th>Heat treatment</th>
<th>Filtration</th>
<th>Level of lactose % of original level</th>
<th>Amount of lactose % w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional indirect UHT</td>
<td>-</td>
<td>Indirect</td>
<td>-</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>Lactose- hydrolyzed direct UHT</td>
<td>+</td>
<td>Direct</td>
<td>+</td>
<td>&lt;1</td>
<td>0.01</td>
</tr>
<tr>
<td>Lactose- hydrolyzed indirect UHT</td>
<td>+</td>
<td>Indirect</td>
<td>+</td>
<td>&lt;1</td>
<td>0.01</td>
</tr>
<tr>
<td>Filtered indirect UHT</td>
<td>-</td>
<td>Indirect</td>
<td>+</td>
<td>60</td>
<td>3</td>
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</tbody>
</table>
Table 2. Overview of the chemical changes in carbohydrates, proteins and Strecker degradation compounds during storage of conventional, lactose-hydrolyzed and filtered UHT milk.

<table>
<thead>
<tr>
<th>Chemical compound</th>
<th>Conventional indirect UHT</th>
<th>Lactose-hydrolyzed direct UHT</th>
<th>Lactose-hydrolyzed indirect UHT</th>
<th>Filtered indirect UHT</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose*</td>
<td>NS (54.18-54.31)</td>
<td>NS (0.278-0.214)</td>
<td>NS (0.249-0.207)</td>
<td>NS (27.30-39.19)</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Galactose*</td>
<td>NS (0.356-0.407)</td>
<td>NS (34.53-27.94)</td>
<td>NS (24.56-27.88)</td>
<td>NS (0.208-0.275)</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Glucose*</td>
<td>ND</td>
<td>NS (41.43-32.66)</td>
<td>NS (29.17-32.84)</td>
<td>NS (0.136-0.148)</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Formate*</td>
<td>(↑) p=0.025 (0.02-0.034)</td>
<td>(↑) p=0.001 (0.007-0.022)</td>
<td>(↑) p=0.001 (0.008-0.02)</td>
<td>NS</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Alanine*</td>
<td>NS (0.019-0.018)</td>
<td>(↑) p&lt;0.001 (0.018-0.026)</td>
<td>(↑) p&lt;0.001 (0.015-0.027)</td>
<td>NS</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Isoleucine*</td>
<td>NS (0.003-0.003)</td>
<td>(↑) p=0.007 (0.007-0.129)</td>
<td>(↑) p&lt;0.001 (0.067-0.154)</td>
<td>NS</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Leucine*</td>
<td>NS (0.001-0.001)</td>
<td>(↑) p=0.015 (0.003-0.033)</td>
<td>(↑) p=0.003 (0.003-0.033)</td>
<td>NS</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Lysine*</td>
<td>NS (0.007-0.016)</td>
<td>(↑) p=0.012 (0.007-0.018)</td>
<td>(↑) p=0.026 (0.007-0.023)</td>
<td>NS</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Methionine*</td>
<td>NS (0.004-0.007)</td>
<td>NS (0.002-0.005)</td>
<td>NS (0.003-0.003)</td>
<td>NS</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Phenylalanine*</td>
<td>ND (↑) p=0.006</td>
<td>(↑) p=0.001</td>
<td>ND</td>
<td>ND</td>
<td>1H NMR</td>
</tr>
<tr>
<td>Substance</td>
<td>Condition</td>
<td>Value (a)</td>
<td>Condition</td>
<td>Value (a)</td>
<td>Condition</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND</td>
<td>(†) p=0.063</td>
<td>(†) p=0.023</td>
<td>ND</td>
<td>¹H NMR</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>ND</td>
<td>(†) p=0.002</td>
<td>(†) p&lt;0.001</td>
<td>ND</td>
<td>¹H NMR</td>
</tr>
<tr>
<td>Valine</td>
<td>NS</td>
<td>(†) p=0.007</td>
<td>(†) p=0.001</td>
<td>NS</td>
<td>¹H NMR</td>
</tr>
<tr>
<td>Total CN</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>HPLC MS</td>
</tr>
<tr>
<td>Glycoprotein</td>
<td>(†) p=0.002</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>(†) p=0.001</td>
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</tr>
<tr>
<td>κ-CN</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>HPLC MS</td>
</tr>
<tr>
<td>αs1-CN</td>
<td>NS</td>
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<td>(†) p&lt;0.001</td>
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<tr>
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<td>(†) p=0.004</td>
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<td>B-CN</td>
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<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.003</td>
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<tr>
<td>β-LG</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>(†) p&lt;0.001</td>
<td>HPLC MS</td>
</tr>
<tr>
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<td>NS</td>
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<tr>
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<td>NS</td>
<td>GC-MS</td>
</tr>
<tr>
<td>Compound</td>
<td>Start Concentration</td>
<td>End Concentration</td>
<td>Method</td>
<td></td>
<td></td>
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<td>-------------------</td>
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<td></td>
</tr>
<tr>
<td>2-ethylfuran†</td>
<td>(†) p=0.005</td>
<td>NS</td>
<td>GC-MS</td>
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<td></td>
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<td>(0.59-0.68)</td>
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<tr>
<td>2-pentylfuran†</td>
<td>NS ((†) p=0.053)</td>
<td>NS</td>
<td>GC-MS</td>
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<tr>
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<td>(0.99-1.07)</td>
<td>(0.88-0.94)</td>
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<tr>
<td>2-methylfuran†</td>
<td>ND</td>
<td>ND</td>
<td>GC-MS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Average start and end concentration, [mM]

# Average start and end concentration, % intact protein

□ Average start and end concentration, [µg/kg]

a,b,c Indicate a significant difference in concentration between the milk types at storage day 270, p<0.05

ND Not detected

NS Not significant

(†) Indicates a significant increase during storage

(↑) Indicates a significant decrease during storage
Table 3. Amino acids identified using $^1$H NMR spectroscopy and their involvement in formation of Strecker degradation aldehydes identified using GCMS

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Strecker degradation product</th>
<th>Identified with GCMS</th>
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</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>2-methylbutanal</td>
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</tr>
<tr>
<td>Leucine</td>
<td>3-methylbutanal</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>Methylpropanal</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>Acetaldehyde $\rightarrow$ 2-methylfuran, 2-pentylfuran</td>
<td>X</td>
</tr>
<tr>
<td>Lysine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>Methional $\rightarrow$ Dimethyl disulfide and dimethyl trisulfide</td>
<td>X</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Benzaldehyde, 2-methylfuran</td>
<td>X</td>
</tr>
<tr>
<td>Tyrosine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
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</tbody>
</table>
Figures

Figure 1
Figure 2.
Figure 3.
Figure 4.
Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk


Submitted to Food Research International
Title: Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk

Article Type: Research Article

Keywords: Bitter taste; Lactose-hydrolyzed milk; UHT; Sensory descriptive analysis; GC-MS; Stale flavor

Corresponding Author: Dr H.C. Bertram,

Abstract: Storage-induced changes are known to be more prominent in lactose hydrolyzed (LH) milk compared to conventional milk. Therefore the present study aimed at identifying off-flavors resembling from formation of volatiles during storage of ultra high temperature treated (UHT) LH milk and conventional UHT milk. Further, the influence of heat processing, indirect or direct, on UHT LH milk was also examined. Storage-induced changes in sensory attributes, volatiles and primary amines were investigated during a four months period. Conventional UHT milk (with 5 % lactose) processed using indirect heat treatment (CONVI) and two types of UHT lactose-hydrolyzed (LH) milk (with less than 0.01% lactose) produced using either direct heat treatment (LHD) or indirect heat treatment (LHI) were represented in the study. Sensory descriptive analysis showed that fresh samples of CONVI, LHI and LHD differed in sensory properties and the samples could be differentiated according to boiled and stale aroma as well as color saturation. Differentiation of the fresh samples based on the volatile GC-MS profile was not achievable. During storage, samples developed differently with respect to sensory characteristics, volatiles and the amount of primary amines. Partial least squares models (PLS1) including only methyl ketones and aldehydes showed that 2-butanone, 2-pentanone, 2-heptanone, 2-nonanone, heptanal, octanal and nonanal predicted stale flavor. Bitter taste, on the other hand, correlated with the amount of primary amines (Pearson's correlation, r²=0.71). This finding indicates that storage-induced changes in sensory characteristics and chemical composition depend on both differences in lactose content and the applied heat processing.
Dear Editor

Enclosed please find the manuscript entitled “Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk”.

The authors believe that the manuscript is suitable for publication in Food Research International. The manuscript concerns the investigation of off flavor development in lactose hydrolyzed milk. Off flavor development was measured by sensory descriptive analysis, GC-MS and fluorescamine assay. The aim of the study was to elucidate the influence of enzymatic lactose hydrolysis, heat processing and storage on sensory characteristics on milk stored for up to 4 months. Changes in the sensory characteristics were related to changes in volatiles with a special focus on the relationship between methyl ketones and aldehydes and stale flavor. Further, the relationship between the degree of proteolysis and bitter taste was examined. The study showed that changes in sensory characteristics and volatiles were dependent on both milk type and differences in heat processing which is valuable knowledge in the process optimization of lactose hydrolyzed milk and for a future development of new lactose hydrolyzed products. We believe that the topic fits well with the aims and scope of Food Research International.

We hope that you will consider the manuscript for publication and we are looking forward to be hearing from you.

On behalf of the authors,
Sidsel Jensen and Hanne Christine Bertram
Highlights

- Sensory characteristics of stored lactose-hydrolyzed and conventional milk differ
- Off-flavor development in lactose-hydrolyzed milk depends on UHT processing method
- Development of volatiles depends on milk type and UHT processing method
- The bitter taste of milk correlates with the content of primary amines
Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk

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Abstract
Storage-induced changes are known to be more prominent in lactose hydrolyzed (LH) milk compared to conventional milk. Therefore the present study aimed at identifying off-flavors resembling from formation of volatiles during storage of ultra high temperature treated (UHT) LH milk and conventional UHT milk. Further, the influence of heat processing, indirect or direct, on UHT LH milk was also examined. Storage-induced changes in sensory attributes, volatiles and primary amines were investigated during a four months period. Conventional UHT milk (with 5 % lactose) processed using indirect heat treatment (CONVI) and two types of UHT lactose-hydrolyzed (LH) milk (with less than 0.01% lactose) produced using either direct heat treatment (LHD) or indirect heat treatment (LHI) were represented in the study. Sensory descriptive analysis showed that fresh samples of CONVI, LHI and LHD differed in sensory properties and the samples could be differentiated according to boiled and stale aroma as well as color saturation. Differentiation of the fresh samples based on the volatile GC-MS profile was not achievable. During storage, samples developed differently with respect to sensory characteristics, volatiles and the amount of primary amines. Partial least squares models (PLS1) including only methyl ketones and aldehydes showed that 2-butanone, 2-pentanone, 2-heptanone, 2-nonanone, heptanal, octanal and nonanal predicted stale flavor. Bitter taste, on the other hand, correlated with the amount of primary amines (Pearson’s correlation, $r^2=0.71$). This finding indicates that storage-induced changes in sensory characteristics and chemical composition depend on both differences in lactose content and the applied heat processing.

Keywords: Bitter taste; Lactose-hydrolyzed milk; UHT; Sensory descriptive analysis; GC-MS; Stale flavor
**Chemical compounds**

2-butanone (PubChem CID: 6569); 2-pentanone (PubChem CID: 7895); 2-heptanone (PubChem CID: 8051); 2-nonanone (PubChem CID: 13187); heptanal (PubChem CID: 8130); octanal (PubChem CID: 454); nonanal (PubChem CID: 31289)
1. Introduction

The prevalence of adult-type hypolactasia is widespread throughout the world (Itan, Jones, Ingram, Swallow, & Thomas, 2010). Historically, hypolactasia frequency is lowest in Northwestern Europe but the frequency is increasing probably due to an increase in immigration of citizens with adult-type hypolactasia (Almon, Engfeldt, Tysk, Sjöström, & Nilsson, 2007). Consequently, the sale of lactose-free dairy products tripled in Europe from 2007 to 2011 and it is also expected to double in the period 2012 to 2016 (Astley, 2012). However, according to a recent global market report the most significant opportunities exist in markets that have a prevalence of lactose intolerance and where dairy consumption is rising, such as in Asia and Latin America (Zenith International, 2012).

Traditionally, lactose-hydrolyzed (LH) milk is sold as UHT-treated products, and they have been claimed to have a shorter shelf-life due to limited chemical stability compared to conventional UHT milk (Messia, Candigliota, & Marconi, 2007). The chemical instability is expected to result in perceptible alterations of the product reducing the overall product quality (Lawless, 1995).

Unaltered sensory characteristics of lactose-hydrolyzed UHT milk during storage can only be achieved, if the chemical and physical processes from which the off-flavors originate are prevented. Vallejo-Cordoba and Nakai (1994) found that the shelf-life of pasteurized milk as determined by sensory evaluation was better predicted by analysis of volatiles than by bacterial count. In UHT-treated milk, the severity of heating applied to the milk and the concentration of dissolved oxygen in the milk immediately after processing depends on the type of UHT processing and are the two most important factors contributing to off-flavor formation (Datta, Elliott, Perkins, & Deeth, 2002). The most commonly applied UHT processing is indirect heat processing where the milk and the heating source are physically separated at all times. Due to a shorter heating and cooling time, direct UHT processing, where steam is injected or infused directly into the milk stream, is more gentle, but also a more costly method compared to indirect UHT processing (Elliott,
The level of dissolved oxygen in direct UHT processed milk is also lower than in milk exposed to indirect UHT processing (Datta et al., 2002).

Recently, numerous papers encompassing sensory analysis of conventional UHT milk have been reported (Jokar & Karbassi, 2011; Lorenzen et al., 2011; Ochi et al., 2010). However, only a few of these sensory studies addresses lactose-hydrolyzed milk (Adhikari, Dooley, Chambers, & Bhumiratana, 2010; Tossavainen, 2008). Tossavainen (2008) investigated sensory differences between conventional and lactose-hydrolyzed UHT milk stored for 10 weeks at 22°C and found no perceptual difference between the two milk types. The study revealed that the first quality defects occurred after 6 weeks of storage and encompassed cooked, heated and burned flavor followed by both an atypical and a bitter taste. In contrast, Adhikari et al. (2010) found a higher intensity of sweet taste, chalkiness and cooked and processed flavors in fresh samples of lactose-free ultra-pasteurized milk as compared with pasteurized conventional milk.

In total, more than 400 volatiles have been associated with milk. During storage at room temperature proteins, lipids and sugars are involved in different chemical reactions in UHT-processed milk, and these chemical changes are reported to change the sensory characteristics (Singh, Ruhil, Jain, Patel, & Patil, 2009; Valero, Villamiel, Miralles, Sanz, & Martinez-castro, 2001). The Maillard reaction, which is a heat-induced condensation reaction involving the free aldehyde group of reducing sugars and free amines in proteins and peptides, occurs during the UHT treatment of milk and continues to progress during storage (Kato, Matsuda, Kato, & Nakamura, 1988; Pellegrino, De Noni, & Resmini, 1995). More Maillard reaction products are formed during storage of LH milk compared to conventional milk (Jansson et al., 2014b; Messia et al., 2007). Formation of stored, stale and rancid flavors have been found to take place during storage of UHT milk and this has been assigned to increased concentrations of methyl ketones and aldehydes, which originate from the ongoing Maillard reaction and lipid oxidation (Jeon, Thomas, & Reineccius, 1978; Rerkrai,
Jeon, & Bassette, 1987). Proteolysis in UHT milk leads to exposure of free amines and this may likewise increase formation of Maillard reactions products and thus facilitate the formation of stored, stale and rancid flavors. Furthermore, bitter peptides formed during proteolysis may contribute to an increased bitterness. McKellar, Froehlich, Butler, Cholette, & Campbell (1984) found a correlation between the degree of proteolysis and bitter taste in UHT milk.

In order to obtain a better understanding of how storage-induced changes differ in conventional and LH milk, the objective of the present study was to elucidate the influence of enzymatic lactose hydrolysis, heat processing and storage on sensory characteristics on milk stored for up to four months. Changes in the sensory characteristics were related to changes in volatiles with a special focus on the relationship between methyl ketones and aldehydes and stale flavor. Further, the relationship between the degree of proteolysis and bitter taste was examined.

2. Materials and methods

2.1 Chemical standards

Decane (99 %), tetradecane (99 %), dodecane (99 %), 2-methylbutanal (95 %), hexanal (98 %), octanal (99 %), nonanal (95 %), decanal (98 %), undecanal (97 %), 2-butanone (99 %), 2-pentanone (99 %), 2-hexanone (99.5 %), 2-heptanone (98 %), 2-octanone (98 %), 2-nonanone (99 %), 2-undecanone (99 %), 6-methyl-5-heptene-2-one (99 %), 6,10-dimethyl-5,4-undecadien-2-one (97 %), dimethyl disulfide (98 %), dimethyl trisulfide (98 %), 4-methyl-1-pentanol (97 %), toluene (99.5 %), styrene (99 %), 2-pentylfuran (97 %), caffeine (Ph Eur) and calcium glycerophosphate hydrate (Ph Eur) were all purchased from Sigma-Aldrich.
Inc. (Stenheim, Germany). Dimethyl sulfoxide (99 %) was purchased from Serva electrophoresis GmHB (Heidelberg, Germany).

2.2 Milk samples

Three types of milk were produced at a commercial Danish dairy plant (Arla Foods amba, Esbjerg, Denmark). Conventional UHT milk (with 5 % lactose) processed using indirect heat treatment (CONVI) and two types of UHT lactose-hydrolyzed (LH) milk (with less than 0.01 % lactose) produced using either direct heat treatment (LHD) or indirect heat treatment (LHI) (Table 1). In LHD and LHI products the reduction in lactose was obtained by a combination of ultra-filtration (to approximately 60 % of the original concentration) prior to heat processing and enzymatic hydrolysis by addition of β-galactosidase after UHT processing.

LHD milk was produced by steam injection directly into the milk stream allowing the milk to instantly reach a temperature of 140-145 °C. This temperature was kept for a few seconds and subsequently the milk was cooled by flash cooling. LHI and CONVI milk were produced using a tube heat exchanger where the milk was heated to a temperature of 140-145°C for a few seconds and after that cooled by a slow cooling process (Tetra Pak, 2003, Chapter 9). Subsequently the milk was tapped and packed aseptically in 1-L packages. Milk was produced at three different occasions: September 2012, October 2012 and February 2013 and analyzed simultaneously in February 2013 resulting in fresh milk and milk stored for three and four months at the date of analysis. During storage, the milk was kept in an air-conditioned room at 22°C.

2.3 Sensory descriptive analysis

The milk used for sensory descriptive analysis (DA) was stored in a refrigerator approximately 24 hours prior to analysis. Two hours before the sensory session approximately 20 mL milk was poured into
transparent plastic glasses (total volume 30 mL) from ABENA (Aabenraa, Denmark) labeled with a random three-digit number. The glasses were sealed with a frosted white plastic lid and placed in a thermostat controlled heat cabinet from Termaks (Bergen, Norway) adjusted to a temperature of 15 °C for 1-2 hours. Samples were removed from the heat cabinet and placed at room temperature 15 min before the sensory session.

An established and trained sensory panel was used for DA. The panel consisted of ten assessors (eight females/two males, aged from 29 to 59) with one to seven years of experience in evaluation of food products and all with prior experience in DA of milk. During two training sessions (2 hours each) assessors were presented for reference samples deliberately designed to represent sensory attributes relevant for stored UHT milk (sulfurous, cabbage-like, cardboard-like, stale, bitter, chalky, sweet/caramel-like, rice pudding-like and popcorn-like) together with extreme samples from the actual test set (LHD and CONVI, 0 months and LHI and CONVI 4 months). Initially, the sensory panel agreed upon a list of 12 sensory attributes divided into four main categories: appearance, aroma (nasal perception), flavor/taste (retro nasal perception/oral perception) and mouthfeel (Figure 1A-L), and afterwards assessors evaluated a set of extreme samples in the sensory booths. After each training session, assessors received feedback on their performance with the aim of improving and standardizing the panel's discriminating power.

The DA was performed in a sensory evaluation laboratory fulfilling requirements settled by the American Society for Testing and Materials (ASTM, 1986) and the analysis was conducted in agreement with specifications provided by the International Organization for Standardization (ISO, 1993). To test the robustness of the DA the fresh sample of LHD was included twice in the analysis. Samples were analyzed in quadruples over two test days (two replicates each day).

2.4 Analysis of volatiles
Sampling and analysis of volatiles was analyzed by dynamic headspace gas chromatography mass spectrometry (GC-MS) performed according to the optimized method described previously by Jansson et al. (2014a). Three replicates of each type of milk from the same 1-L package were analyzed at the same days as the sensory analysis. Triplicates of CONVI stored for four months from two different packages were encompassed in the analysis to verify the reproducibility of the method. An MS database (NIST MS search version 2.0, 2011) was used to identify 27 volatiles. In addition, all of the volatiles were verified by comparison of mass spectral data and retention times of authentic standards. Twelve calibrants (2-methylfuran (6.6 min), 2-methylbutanal (7.4 min), 2-ethylfuran (8.2 min), 2-pentanone (10.7 min), dimethyl disulphide (DMDS) (14.9 min), hexanal (19.2 min), 2-heptanone (31.9 min), 2-pentylfuran (36.0 min), 2-octanone (36.8 min), dimethyl trisulphide (DMTS) (40.1 min), 2-nonanone (41.9 min) and nonanal (42.0 min)) were used for quantification of volatiles belonging to the same class and/or compounds eluting in the same retention time window. Standard curves, made in duplicates, were prepared using ultra-filtered UHT milk and sampled and analyzed in a manner resembling the procedure used for analysis of the actual milk samples. The chromatogram of the pure milk was subtracted from the recorded chromatograms. All concentrations were corrected in accordance to the response factor of an internal standard (4-methyl-1-pentanol). Limit of detection (LOD) was calculated from the standard error of the Y-intercept and the slope of the linear regression (Dolan, 2009).

2.5 Determination of primary amines by fluorescamine assay

The level of primary amines in the milk was determined according to the method of Dalsgaard, Nielsen, & Larsen (2007). Milk was mixed with an equal volume of 24 % trichloroacetic acid and put on ice for 30 min in order to accelerate protein precipitation. The precipitated mixture was centrifuged (17,000 g) for 20 min. Part of the supernatant, 37.5 μL, was added to 1130 μL 0.1M borate buffer and 375 μL of a fluorescamine solution (0.2 mg/mL fluorescamine in water free acetone). After 18 min, 250 μL of the resulting solution
was transferred to a 96-well micro plate and fluorescence was measured using a Synergy 2 Multi-Mode Microplate Reader (Holm & Halby, Denmark) with excitation and emission wavelengths of 400 nm and 485 nm. Quantification was achieved using an external leucine standard curve made in 1 mM HCl (0.5 – 3 mM leucine) and results were given as leucine equivalents.

### 2.6 Statistical analysis

Statistical analyses were carried out using the freeware programs PanelCheck v. 1.4.0 (www.PanelCheck.com) and R v. 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria). PanelCheck was used for assessor evaluations and for sensory product differences tested by Bonferroni least significant difference (LSD) while R was used on sensory data for a complete analysis of linear mixed effect models (Kuznetsova, Brockhoff, & Christensen, 2013) with assessor and replicate as random effects. Analysis of variance (ANOVA) was applied to investigate significant differences of volatiles between milk samples. Statistical significances were defined at $p \leq 0.05$. Compounds with concentrations below LOD were set to zero. Partial least squares regression (PLS1) was performed using Simca software version 13.0 from Umetrics (Umea, Sweden) in order to predict stale flavor on basis of mean data from the analysis of selected volatiles. All data was scaled to unit-variance prior to analysis and full cross validation was applied. Person’s correlation coefficient ($r^2$) was calculated using R to determine the linear relationship between bitter taste and the content of primary amines.

### 3. Results and discussion

#### 3.1 Sensory descriptive analysis (DA)

Figure 1 shows the development during storage for each of the twelve attributes included in the DA: boiled milk aroma (A), popcorn aroma (B), stale aroma (C), color saturation (D), boiled milk flavor (E), popcorn...
flavor (F), stale flavor (G), caramel flavor (H), creamy flavor (I), sweet taste (J), bitter taste (K) and fatty mouthfeel (L). No significant differences were identified between the two fresh samples of LHD milk originating from the same 1-L package for any of the tested attributes confirming the robustness of the DA. Because the two samples were perceived as being identical, the mean intensity score for each repetition of these two samples was used in the further statistical models.

Fresh samples of milk could be differentiated according to boiled milk aroma (Figure 1A), color saturation (Figure 1D) and fatty mouthfeel (Figure 1L). Cooked/boiled milk aroma in milk was associated with an increase in the formation of sulfur-containing compounds most probably arising from the thermally denaturation of serum proteins (de Wit and Nieuwenhuijse, 2008) and, thus, it was unexpected to find that fresh LHD, representing the sample with the lowest heat load, had a higher intensity of boiled milk aroma (Figure 1A) than both CONVI and LHI. However, the result is supported by Ochi et al. (2010) who likewise found a more intense overall aroma, butter aroma and cooked flavor in direct UHT processed milk as compared to samples exposed to indirect UHT treatment. In other studies a higher intensity of cooked and processed flavor and a sweeter taste was found in fresh LH milk compared with fresh samples of conventional milk (Adhikari et al., 2010; Jokar & Karbassi, 2011) or no observable differences were recognized (Tossavainen, 2008). After three months of storage, CONVI had a higher degree of color saturation (Figure 1D) than LHI, a more intense boiled milk flavor (Figure 1E), cream flavor (Figure 1I) and a more fatty mouthfeel (Figure 1L) compared to LHI and LHD whereas LHI was evaluated to have a more stale flavor (Figure 1G) compared to CONVI. Color saturation (Figure 1D) was significantly lower in fresh and three months old LHD than in similar samples of CONVI, while the difference disappeared after 4 months of storage. This finding indicates that the type of heat processing was decisive for the color saturation of milk the first three months of storage, possibly due to different effects on the degree of heat-induced protein denaturation. After four months of storage, differences in boiled aroma (Figure 1A), creamy flavor (Figure
1) and fatty mouthfeel (Figure 1L) were identified. LHI was evaluated to have a more intense boiled milk aroma (Figure 1A) than LHD whereas CONVI was judged to have a higher intensity of creamy flavor (Figure 1J) than both LHI and LHD and to provide a more fatty mouthfeel (Figure 1L) than LHD.

Seven attributes obtained a significant interaction effect between milk type and storage: boiled milk aroma ($p \leq 0.0001$, Figure 1A), stale aroma ($p \leq 0.0001$, Figure 1C), color saturation ($p \leq 0.0001$, Figure 1D), boiled milk flavor ($P = 0.05$, Figure 1E), stale flavor ($p \leq 0.0001$, Figure 1G), creamy flavor ($p \leq 0.0001$, Figure 1I) and bitter taste ($0.05$, Figure 1K).

FIGURE 1

Tossavainen (2008) identified an increase in cooked, heated and burned flavor after six weeks of storage as the first indicator of storage-related quality defects in LH milk. In this study, cooked, heated and burned flavor were associated with boiled milk aroma (Figure 1A) and flavor (Figure 1E), and LHI and CONVI milk showed an increase in intensity of boiled milk aroma (Figure 1A) from fresh to 3 months of storage, whereas a decrease in this attribute was observed for LHD during storage. Boiled milk flavor (Figure 1E) did not increase in any of the tested milk types during storage. The second indicator of storage-related quality defects that Tossavainen (2008) observed was a combination of an atypical and a bitter taste. These quality defects can be associated with stale flavor (Figure 1G) and bitter taste (Figure 1K) in the present study, which both showed a significant interaction effect between milk type and storage. This could indicate that development of these two attributes was dependent on the milk type and that a general conclusion about the development across milk types should be made with caution. Stale flavor originates from volatiles formed via the Maillard reaction and lipid oxidation (Jeon et al., 1978; Rerkrai et al., 1987),
both of which are catalyzed by heat. However, no significant effect of direct versus indirect heating could be observed in the fresh milk, whereas during storage stale flavor increased the first three months of storage in LHD and then settled. In contrast, LHI and CONVI demonstrated the lowest amount of stale flavor after three months of storage (Figure 1G).

Bitter taste is known to be associated to proteolytic activity in LH milk (Tossavainen, 2008). In the present study bitter taste likewise increased in lactose reduce milk (LHD and LHI) while it remained constant in CONVI. In addition, there was a significant decline in sweet taste for all milk types (Figure 1J) during storage which could indicate a reduction in free sugars available, e.g. lactose, galactose and glucose, due to ongoing Maillard reactions during storage (Chávez-Servín, Romeu-Nadal, Castellote, & López-Sabater, 2006) or alternatively because of a masking effects due to an increase in bitter taste that is known to reduce the perceived sweet taste (Beck, Jensen, Bjoern, & Kidmose, 2014). The masking effect is more likely because Jansson et al. (2014b) could not analyze changes in the content of simple sugars in UHT processed LH milk during 9 months of storage. Fatty mouthfeel (Figure 1L) was the only attribute where a significant effect of milk type was observed, with CONVI being the milk type with the most profound fatty mouthfeel (Figure 1L). This fact might be due to a smaller milk fat globule size in the LH milk types as a result of an additional membrane filtration processing, however, this hypothesis needs to be further investigated.

3.2 Relationship between the chemical composition and sensory characteristics

The composition of the volatile components in the milk samples is responsible for the perceived aroma. Volatiles present in levels above the sensory threshold (Table 2) can be expected to be important contributors to the aroma of food products, however, also volatiles below the sensory threshold will possibly influence perceived aroma due to mixture enhancement (Jeon et al., 1978). Only minor differences in the composition of volatiles between the duplicate CONVI samples stored for four months were found by
GC-MS analysis and therefore means were used for statistical calculations. Of the 27 compounds verified by authentic standards, a total of 17 compounds: 2-butanone, 2-methylbutanal, 2-pentanone, decane, toluene, DMDS, 2-hexanone, hexanal, 2-heptanone, heptanal, 2-octanone, octanal, DMTS, 2-nonanone, nonanal, tetradecane and decanal, occurred in concentrations equal to or higher than the LOD in one or more of the tested milk types (Table 2). The presence of these compounds in milk was in agreement with previous GC-MS studies conducted on milk (Contarini et al., 1997; Jansson et al., 2014a; Valero et al., 2001; Vazquez-Landaverde, Velazquez, Torres, & Qian, 2005).

Intriguingly, no significant differences between fresh samples of milk were observed for any of the detected volatiles and thus the GC-MS analysis failed to differentiate and explain why fresh LHD milk was perceived to have a higher intensity of boiled milk aroma (Figure 1A) compared to CONVI and LHI. After three months of storage, CONVI had a significantly higher content of 2-butanone and hexanal and a significantly lower content of DMDS than LHD and LHI and a significantly lower content of 2-methylbutanal, DMTS and tetradecane than LHD (Table 2). The lower content of 2-methylbutanal in CONVI stored for three months compared to both types of LH milk was a strong indicator of a more slow progression of the Maillard reaction in CONVI during storage, considering 2-methylbutanal being a typical Maillard reaction product in milk (Jansson et al., 2014b). Oxidative degradation of methionine leads to a higher content of DMDS and DMTS (Ballance, 1961). Consequently, the higher content of dissolved oxygen found in indirect UHT processed milk compared to direct UHT processed milk (Datta et al., 2002) must be expected to result in generation of a higher concentration of DMDS and DMTS. However, concurrently a higher level of dissolved oxygen is reported to cause a more rapid loss of sulphydryl compounds (Thomas, Burton, Ford, & Perkin, 1975), which may explain why the concentration of DMDS and DMTS cannot be linked to heat processing method and may also be the reason that no differences in boiled milk aroma were evident between milk samples stored for three months. After four months of storage, significantly lower contents of toluene, hexanal, 2-heptanone and 2-nonanone were identified in LHI compared to CONVI and LHD as well as
significantly lower contents of 2-butanone, 2-pentanone and 2-octanone than CONVI. LHD stored for four months had a significantly lower concentration of DMDS and DMTS compared to LHI (Table 2).

TABLE 2

Eleven of the identified volatiles exhibited a significant interaction between storage and milk type: 2-butanone \((P=0.007)\), 2-pentanone \((P=0.03)\), toluene \((P=0.0005)\), DMDS \((P=0.0003)\), 2-hexanone \((P=0.006)\), hexanal \((P=0.0002)\), 2-heptanone \((P=0.005)\), 2-octanone \((P=0.007)\), DMTS \((P=0.01)\), 2-nonanone \((P=0.0005)\), and tetradecane \((P=0.0003)\), supporting the results from the sensory descriptive analysis, which showed that the changes in the sensory characteristics during storage highly depended on milk type and heating process.

As LH milk has been found to have a shorter shelf-life than conventional milk (Messia et al., 2007), and as previous studies have associated storage-related off-flavor in UHT milk with increasing concentrations of methyl ketones and aldehydes (Jeon et al., 1978; Rerkrai et al., 1987), the development in methyl ketones and aldehydes during storage were examined in the three types of milk. It was investigated if some of the methyl ketones and aldehydes were better predictors of the development of stale flavor than others. Stale flavor was the attribute from the present study assumed to be closest related to the storage related off-flavor described in the abovementioned studies. For this purpose, PLS1 regression was conducted only including methyl ketones (six volatiles) and aldehydes (six volatiles) as X-variables and stale flavor as Y-variable. The optimal number of PC’s in the prediction of stale flavor was four which resulted in a cumulative cross-validation variance in Y \((Q_{2Y\_cum})\) of 0.81, meaning that the methyl ketones and aldehydes included in the model were able to predict 81 % of the variance related to stale flavor. The model explained 94 % of the variance related to methyl ketones and aldehydes \((R_{2X\_cum})\). Figure 2 displays the PLS1 score scatter plot (A) and the corresponding loading plot (B) including volatiles (X-
variables) and stale flavor (Y-variable) for PC1 and PC2. From the figure it was obvious that fresh samples of milk and milk stored for three months differ in the content of methyl ketones and aldehydes depending on milk type. Fresh CONVI and CONVI stored for three months are positioned in the upper and lower left quadrant of the PLS1 score plot while fresh LHD and LHD stored for three months were positioned in the lower left and the upper right quadrant. Fresh LHI and LHI stored for three months were positioned in the lower right and the upper left quadrant. All samples stored for four months were positioned in the right side of the score plot (Figure 2A). Samples positioned in the lower left quadrant were characterized by a high concentration of 2-butanal, hexanal and decanal while samples positioned in the upper right quadrant have a higher concentration of 2-butanal, 2-pentanal, 2-heptanal, 2-nonanal, heptanal, octanal and nonanal. These compounds were concurrently found to be the best predictors of stale off-flavor and it appears to be the combination of these compounds, more than the individual volatiles that gave rise to the stale flavor.

FIGURE 2

Generally, the concentration of ketones increased in CONVI and LHD milk during storage while a decrease in concentration was seen for LHI milk (Table 2). Three of the six identified aldehydes showed a significant increase in concentration during storage: 2-methylbutanal ($P = 0.002$), octanal ($P = 0.003$) and decanal ($P = 0.01$) and an interaction effect between milk type and storage was only seen for hexanal. Overall, these results indicate that the formation of aldehydes during storage was independent of milk type while the generation or loss of ketones highly depends on the type of milk. Further, both methyl ketones and aldehydes influenced stale flavor.
A consistent quality defect associated with stored UHT processed milk upon storage is bitter taste (McKellar et al., 1984; Tossavainen, 2008). In this study, the level of primary amines was significantly correlated with the intensity of bitter taste ($R^2 = 0.71$, Figure 3). This has previously been observed in conventional milk (McKellar et al., 1984), but has not previously been elucidated in LH milk underlying the importance of the results obtained in this study. The level of primary amines increased more progressively in LH compared to CONVI (Table 3) indicating that proteolysis was found to be more pronounced in LH milk compared to CONVI. As the level of primary amines and the bitter taste was correlated, it is most probably enhanced proteolysis that is responsible for the observed development of bitter taste in LH milk. Since there should be no variation in the level and activity of endogenous enzymes between the investigated milk types we expect that the increased in proteolysis in LH milk to be the result of proteolytic side-activity from the β-galactosidase enzyme applied to hydrolyze the lactose in the production of LH milk.

4. Conclusions

Results from the sensory descriptive analysis showed that fresh samples of CONVI, LHD and LHI differed in the intensity of boiled and stale aroma as well as color saturation whereas a differentiation of the fresh milk samples based on the volatile profile was not achievable. During storage sensory characteristics and volatiles developed differently depending on milk type and heat processing. Intriguingly, stale flavor proved not to be a strictly storage-related off-flavor as the intensity of stale flavor was found to be highest in fresh CONVI of all tested samples. 2-Butanone, 2-pentanone, 2-heptanone, 2- nonanone, heptanal, octanal and nonanal were found to be strong predictors of stale flavor in tests only including methyl ketones and aldehydes. Based on these results, it can be concluded that formation of aldehydes was independent of milk type while the ketone level highly depended on milk type. Bitter taste could be related to the level of primary amines which were generally higher in LH milk compared to CONVI and more pronounced in
samples stored for four months compared to fresh samples and samples stored for three months. The output from this study is valuable knowledge in the process optimization of LH products and for a future development of new LH products.

**Acknowledgement**

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References


Figure 1: Intensity of sensory attributes in different types of milk (LHD: lactose-hydrolyzed milk exposed to direct UHT treatment; LHI: lactose-hydrolyzed milk exposed to indirect UHT treatment; CONVI: conventional milk exposed to indirect UHT treatment) during a storage period of four months. Lower case letters indicate statistical differences between different milk at the same storage time ($P \leq 0.05$).
Figure 2: (A) Scores and (B) Loadings from PLS1 regression with methyl ketones (6) and aldehydes (6) detected by GC-MS as X-variable and stale flavor determined from sensory analysis as Y-variable. Different samples of milk (LHD: lactose-hydrolyzed milk exposed to direct UHT treatment (dark gray); LHI: lactose-hydrolyzed milk exposed to indirect UHT treatment (light grey); CONVI: conventional milk exposed to indirect UHT treatment (black)) were for up to four months.
Figure 3: Correlation plot of the level of primary amines (N-terminals) (X-axis) and bitter taste intensity of three different milk types (LHD: lactose-hydrolyzed milk exposed to direct UHT treatment (dark grey); LHI: lactose-hydrolyzed milk exposed to indirect UHT treatment (light grey); CONVI: conventional milk exposed to indirect UHT treatment (black)) stored for up to four months.
Table 1: Specifications of milk samples

<table>
<thead>
<tr>
<th>Milk</th>
<th>Storage time [months]</th>
<th>Type of heat treatment</th>
<th>Amount of lactose [% w/w]</th>
<th>Amount of fat [% w/w]</th>
<th>Amount of protein [% w/w]</th>
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</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>1, 3 and 4</td>
<td>Indirect</td>
<td>5</td>
<td>1.5</td>
<td>3.5-3.6</td>
</tr>
<tr>
<td>Lactose-hydrolyzed</td>
<td>0, 3 and 4</td>
<td>Direct</td>
<td>0.01</td>
<td>1.5</td>
<td>3.5-3.6</td>
</tr>
</tbody>
</table>

*Referred to as fresh, *Tested after 1 week of storage
**Table 2**

Table 2: Content [ng·g⁻¹ of milk, ppb] of individual volatiles in different types of milk (LHD: lactose-hydrolyzed milk exposed to direct UHT treatment; LHI: lactose-hydrolyzed milk exposed to indirect UHT treatment; CONVI: conventional milk exposed to indirect UHT treatment) differing with respect to storage time together with odor detection threshold of each volatile. Different letters indicate significant differences (P ≤ 0.05) between milk samples of equal storage time.

<table>
<thead>
<tr>
<th>Milk type</th>
<th>Odor</th>
<th>Threshold [ppb]</th>
<th>CONVI</th>
<th>LHD</th>
<th>LHI</th>
<th>CONVI</th>
<th>LHD</th>
<th>LHI</th>
<th>CONVI</th>
<th>LHD</th>
<th>LHI</th>
<th>CONVI</th>
<th>LHD</th>
<th>LHI</th>
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<td></td>
<td></td>
<td>Fresh</td>
<td>3 months</td>
<td>4 months</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>2-butanol</td>
<td>Sweet, apricot</td>
<td>440³</td>
<td>43.57</td>
<td>97.02</td>
<td>116.69</td>
<td>144.25b</td>
<td>20.93a</td>
<td>28.09a</td>
<td>181.16b</td>
<td>147.16ab</td>
<td>28.66a</td>
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<td>2-methylbutanal</td>
<td>Malt¹</td>
<td>2⁴</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00a</td>
<td>6.48b</td>
<td>3.81ab</td>
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<td>2-pentanone</td>
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<td>70⁵</td>
<td>15.15</td>
<td>13.81</td>
<td>29.58</td>
<td>22.19</td>
<td>18.02</td>
<td>14.13</td>
<td>40.27b</td>
<td>29.39ab</td>
<td>20.61a</td>
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<tr>
<td>Decane</td>
<td>Alkane¹</td>
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<td>7.96</td>
<td>8.01</td>
<td>9.37</td>
<td>7.88</td>
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<td>9.18</td>
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<td>9.33</td>
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<td>Toluene</td>
<td>Paint¹</td>
<td>330⁷</td>
<td>7.59</td>
<td>18.06</td>
<td>23.18</td>
<td>10.35</td>
<td>11.02</td>
<td>13.03</td>
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<td>29.81b</td>
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<td>DMDS</td>
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<td>1.72</td>
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<td>1.28</td>
<td>0.44a</td>
<td>1.83b</td>
<td>1.86b</td>
<td>1.84ab</td>
<td>0.50a</td>
<td>2.59b</td>
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<tr>
<td>2-hexanone</td>
<td>Ether¹</td>
<td>24⁵</td>
<td>0.00</td>
<td>3.27</td>
<td>12.41</td>
<td>2.67</td>
<td>6.45</td>
<td>3.47</td>
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<td>15.28</td>
<td>10.37b</td>
<td>1.10a</td>
<td>1.42a</td>
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<td>7⁴</td>
<td>231.31</td>
<td>366.45</td>
<td>767.06</td>
<td>351.04</td>
<td>327.80</td>
<td>283.67</td>
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<td>5.33</td>
<td>8.01</td>
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<td>0.00</td>
<td>1.31</td>
<td>1.09</td>
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<td>0.00</td>
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<td>2.81</td>
<td>5.57</td>
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<td>DMTS</td>
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<td>1.86</td>
<td>0.85a</td>
<td>3.05b</td>
<td>1.99ab</td>
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<td>3.21</td>
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<td>6.42</td>
<td>12.27</td>
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¹Burdock, 2010, ²Czerny et al., 2008, ³Flavornet, ⁴2014 and ⁵Nagata, 2014, pp. 122-123
**Table 3**: Level of primary amines (mM) in three different types of milk (LHD: lactose-hydrolyzed milk exposed to direct UHT treatment; LHI: lactose-hydrolyzed milk exposed to indirect UHT treatment; CONVI: conventional milk exposed to indirect UHT treatment) during a storage period of four months. Lower case letters indicate statistical differences (P≤0.05) between milk samples.

<table>
<thead>
<tr>
<th>Category</th>
<th>Storage (months)</th>
<th>N-amino terminals (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONVI</td>
<td>Fresh</td>
<td>0.50e</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.53de</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.71c</td>
</tr>
<tr>
<td>LHD</td>
<td>Fresh</td>
<td>0.56de</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.00ab</td>
</tr>
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<td></td>
<td>4</td>
<td>1.01a</td>
</tr>
<tr>
<td>LHI</td>
<td>Fresh</td>
<td>0.61de</td>
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<tr>
<td></td>
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<td>0.92b</td>
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