ABSTRACT

JO, YEJIN. Flavor and Flavor Chemistry of Fluid Milk. (Under the direction of Dr. MaryAnne Drake).

The flavor of fluid milk plays a key role for consumer acceptance, shelf life and other attributes. Certain flavor(s) in milk may create a barrier to consumer acceptance. Off-flavors in milk can be difficult to describe, therefore identifying and confirming responsible compound(s) can be challenging. Understanding how heat treatment affects the flavor of milk is critical to determine off-flavors and methods to mitigate them in milk and milk based beverages. The objective of this dissertation was to investigate the impact of different thermal treatments on fluid milk flavor and to elucidate off-flavor compounds in ultrapasteurized (UP) milk. The results of this study provide elucidation of specific flavors and flavor development in UP milk and their impact on sensory quality of UP milks.

The first study was conducted to characterize the differences in the flavor and volatile compound profiles of skim and 2% milks subjected to high temperature short time (HTST), UP by direct steam injection (DSI-UP) or indirect heating (IND-UP) using sensory and instrumental techniques. Heat treatments and fat level had distinct effects on sensory profiles of milk as demonstrated by higher overall aroma, cooked, and sulfur/eggy flavors with UP milks compared to HTST milks. DSI-UP and IND-UP milks differed from each other by sulfur/eggy, sweet aromatic and cooked flavor intensities. These findings suggested differences in flavor formation by Maillard reaction and protein denaturation during thermal processing that affect the flavor quality of UP milks and distinct sensory and chemical differences between DSI-UP and IND-UP milks.

In the second study, the approach of manufacturing reformulated skim milk (RSM) by mixing milk protein fractions (casein and serum protein) in different ratios was applied to clarify
the source of volatile sulfur compounds (VSC) in skim milk processed by UP. Elevated hydrogen sulfide and carbon disulfide were documented in RSM with higher proportions of serum protein as a percentage of the total protein compared to RSM and skim milk with 20% serum protein as the percentage of the total milk protein. These two compounds were correlated with distinct eggy and sulfur/burnt off-flavors and were attributed to the serum protein portion of milk protein. The combination of sensory and instrumental methods used in the second study effectively identified the source of sulfur compounds in milk, and further confirmed the contribution of hydrogen sulfide and carbon disulfide to eggy and sulfur/burnt flavors, respectively.

The third study was performed to elucidate the formation of VSC in UP milk via thermal reactions of lactose and lactose degradation products with cysteine or methionine in aqueous and skim milk model systems. A follow-up experiment using $[^{13}\text{C}]$ labeled lactose, glucose, and galactose in mixtures with cysteine or methionine showed that methional and dimethyl disulfide originated from methionine/lactose, whereas the formation of dimethyl sulfide and dimethyl trisulfide were involved with subsequent reactions including oxidation. Carbon disulfide was derived from cysteine degradation alone along with the release of hydrogen sulfide. Although lactose is not a direct precursor of hydrogen sulfide and carbon disulfide, results suggested that lactose may promote the degradation or reaction of cysteine during heat treatment.

The results from this dissertation demonstrate flavor differences of fluid milks processed by HTST compared to UP and demonstrate a clear source and precursors of VSC in fluid skim milk with a specific focus on UP treatment. These findings further provide a practical insight into the formation of VSC in fluid milk and milk based beverages that require UP treatment.
Flavor and Flavor Chemistry of Fluid Milk

by

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Food Science

Raleigh, North Carolina
2019

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**BIOGRAPHY**

Yejin Jo was born in Seoul, South Korea on October 15\(^{th}\), 1985 to Sang Hee Jo and Ju Hee Yun. She grew up in Seoul with parents and one brother. Yejin attended Ewha University, which is one of the oldest colleges in the country, for her undergraduate and received a B.S. degree in Nutrition and Food Science in 2008. During her undergraduate, she achieved a teaching certificate for middle and high school. She began her Master’s degree in Food Science at Ewha University, where she explored instrumental flavor analysis under Dr. Young-Suk Kim. She started a professional career in CJ Foods, which is the biggest corporation in food industry South Korea, and spent 4 years on product development and research of soybean fermentation products and starter screening. She got married to Jason Kim in 2013, and they moved to North Carolina in 2014 for his career in medical diagnostic and her desire in advanced degree. She joined the MAD lab in 2014 and started a Ph.D. program under the direction of Dr. MaryAnne Drake in 2015. Yejin does not have a specific hobby but she likes to travel the world (has been to 13 different countries) and seeks the opportunity to travel again in the near future. Lately, she spends most of time with her dog and son, Robin, who was born in January 2018.
ACKNOWLEDGMENTS

I would like to thank Dr. Drake who has the greatest insights and the widest heart. I was extremely fortunate to meet Dr. Drake as my advisor. I hope I can be like Dr. Drake in the near future inspiring other young scientists. I would also like to appreciate all committee members, Dr. Hanson, Dr. Fogleman, Dr. Johanningsmeier, and Dr. Poole for taking the time and patience to provide your expertise and guidance on this research.

To all previous and current lab members, thank you for your hard work and helping everyone’s projects each other. I have learned many things from each of you and I truly enjoyed every experiences in the lab.

I would also thank my parents who always give me unconditional support and sorry for missing many family events with you!!

Lastly, Jason, thank you for everything you did to support me since we moved to NC and encouraging me to develop myself.
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CHAPTER 1: LITERATURE REVIEW. FLAVOR AND FLAVOR CHEMISTRY OF FLUID MILK

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Introduction

Fluid milk for human consumption is defined as “the lacteal secretion, practically free from colostrum, obtained by the milking of one or more healthy cows” (21 CFR 131.110). Milk has been consumed for over 10,000 years (Curry, 2013) as an important contributor to the daily nutrient intake, representing the most complete single food available (Cadwallader and Singh, 2009). In the United States, milk consumption was started via European immigrants in the early 1600s. Many dairies have produced milk commercially since the 1800s along with cattle breed development for dairy purposes. As the population increased around large metropolitan cities, larger quantities were needed, and milk was required to have an extended shelf life due to the longer distances from the farms to stores and expanded distribution supply chain. Consequently, extended shelf life (ESL) milk has developed with the advantage of relatively longer shelf life of milk (Rysstad and Kolstad, 2006). ESL milks have been available in North America since the early 1960s and became a current industry trend in the U.S. due to the ability to meet supply chain demands (Henyon, 1999). Additionally, ESL milk is important because it brings competitiveness in the beverage market and economic interest to the dairy industry (Chapman et al., 2001). Over the last 3 years, U.S. dairy farms produced about 200 billion pounds of milk annually (USDA-ERS, 2016), supporting agricultural and economic activities via milk manufacturing.

Over the past 40 years, overall fluid milk intake by Americans has dropped about 37%, and a decline in per capita consumption of fluid milk products is expected to continue through the 2020s (USDA-ERS, 2017). At the same time, fluid milk has been faced with challenges: (1) milk competes with other beverages for sales, (2) the number of dairy cows in the U.S. will gradually decline to 8.9 million by 2021, and (3) milk prices are expected to rise after 2018.
There is not sufficient evidence to insist that characteristic flavor contributes to the market decline of fluid milk. However, flavor is one of the most significant parameters impacting the quality of consumer acceptance, shelf life and other attributes of dairy products (Drake et al., 2007a; Kühn et al., 2006). Moreover, certain flavor(s) in milk may create a barrier to consumer acceptance (Lee et al., 2017; Perkins and Deeth, 2001). In other words, flavor determines the acceptability of milk, and desirable flavors can provide pleasant experiences and benefits.

In aspects of flavor and flavor chemistry, two categories are commonly discussed: (1) identifying desirable flavor(s), and (2) determining off-flavor compound(s) (Marsili, 2006). Fresh milk ideally has a bland flavor with a slightly sweet taste, while abused milk has a number of different off-flavors commonly described as cardboard, oxidized, cooked, putrid and fruity (Nursten, 1997; Schiano et al., 2017). Off-flavor development in milk is a troublesome issue because the presence of minute quantities of abnormal compounds can cause significant off-flavors due to its bland flavor. Flavor deterioration and loss also occur via various routes, such as microbial spoilage, nutrient degradation and reactions during processing or storage. As such, it can be challenging to confirm and describe off-flavors, and identify which compound(s) is responsible. Despite these challenges, considerable progress has been made towards understanding the flavor chemistry of fluid milk over the last three decades (Friedrich and Acree, 1998; Cadwallader and Singh, 2009; Jeon et al., 1978; Belitz et al., 2009; Czerny and Shieberle, 2007; Jo et al., 2018). Four categories are generally suggested for flavor formation in milk: (1) cow feed and/or metabolism, (2) degradation of milk nutrients and chemical, enzyme, microbial reactions, (3) processing induced, and (4) packaging or storage conditions (Calvo and Hoz, 1992). To date, thermal processing has been predominantly linked to flavor formation, and can
lead to a series of desirable and undesirable chemical reactions, such as Maillard reactions (MR), protein denaturation, and β-oxidation of fatty acids (Cadwallader and Singh, 2009). A number of studies reviewed these primary reactions in milk, including lipid oxidation, and thermal degradation of lipids and proteins regarding flavor formation (Calvo and Hoz, 1992; van Boekel, 1998; Cadwallader and Singh, 2009).

With the recent approach of integrated analysis between instrumental and sensory-directed flavor analysis (Marsili and Miller, 1998; Marsili and Miller, 2002), interest in specific compounds as processing indicators in fluid milk have increased. For example, sulfur compounds and methyl ketones for cooked flavor (Calvo and Hoz, 1992), methyl ketones and aldehydes for stale flavor (Contraini and Povolo, 2002; Vazquez-Landaverde et al., 2005), and aldehydes for oxidized flavor (Zabbia et al., 2012) have been identified. However, milk flavor remains complex due to the fact that many volatile compounds in milk are present at concentrations below their threshold level, and their contribution to the flavor has not been fully described (MacGibbon and Taylor, 2006). In addition, limited information is available on flavor development with a specific focus on the fundamental milk processing perspective. In other words, a critical aspect in flavor profiling of milk requires the elucidation of flavor formation mechanisms or markers by interpreting and identifying compounds responsible for specific flavors (key aroma compounds and odorants).

This review will describe an overview of flavors and flavor chemistry in fluid milk as well as the sources of flavor development and their precursors, reactions, and mechanisms based on the following hypotheses: (1) thermal treatment of milk has distinct effects on the flavor of milk, and (2) differences between milk pasteurization treatments can develop different flavor pathways. Flavor analysis and its application to milk can suggest various approaches to compare
and characterize flavor, identify flavor formation, and predict flavor profiles regarding the effects of various heat treatments on fluid milk flavor.

Composition of Milk

Milk, a rich source of nutrients, contains approximately 87% water and 13% solids when it comes from the cow. The solids portion of milk contains about 3.7% fat and 9% solids-not-fat. Milk fat carries the fat-soluble vitamins A, D, E, and K. The solids-not-fat portion consists of proteins (primarily casein and serum protein), carbohydrates (mainly lactose), and minerals (calcium and phosphorus). Milk also contains significant amounts of riboflavin (vitamin B\textsubscript{2}) and other water-soluble vitamins. Minor constituents, such as hormones, and enzymes are present at trace levels (Fox, 2009).

Lipids

Milk fat was regarded as the most valuable milk constituent, and milk production and pricing was valued largely based on fat content (Fox, 2009). Among the significant nutrients, milk fat not only can be the main source of a wider range of flavors (Nursten, 1997), but also has a large number of compounds contributing to overall milk aroma and taste (MacGibbon and Taylor, 2006). Milk fat is very complex and exist as a unique emulsion, which occurs as fat globules emulsify in the aqueous phase of milk (Fox, 2009; Jensen, 2002). The fat globules are composed of nonpolar or core lipids such as triglycerides (TG), cholesteryl esters, and retinol esters coated with bipolar materials such as phospholipid, proteins, cholesterol, and enzymes on a layer of membrane called the milk fat globule membrane (MFGM; Jensen, 2002). MFGM prevents the globules from aggregation and coalescence, and protects the fat against enzymatic
action, such as lipolysis (Jensen, 2002). It also acts as a natural emulsion agent (Jensen, 2002). Thus, the stability of fat globules depends on the nature of the MFGM (Lee and Sherbon, 2002). MFGM in milk can be altered by various factors, such as animal breed, environmental factors, and processing conditions (Lee and Sherbon, 2002). It is well known that homogenization and heat treatments (e.g., pasteurization) can alter structure and composition of fat globules and MFGM (Lopez, 2005). Homogenization can be applied to disrupt the native structure of globules into smaller sizes which allows them to stay evenly distributed in milk (Argov et al., 2008). Pasteurized milk does not necessarily need to be homogenized. However, homogenized milk should be pasteurized to inactivate native enzymes that deteriorate fat (lipases) and cause rancidity, which results in off-flavors and reduced shelf life in milk. During homogenization, the natural fat globules (diameter 1-10 µm) are disrupted into small globules (diameter < 1 µm) and the surface area is increased by more than ten-fold, which alters the structure of the native MFGM and modifies the composition of the membrane (Sharma and Dalgleish, 1993; Jensen, 2002). Consequently, the large amount of the surface area of milk fat globules is newly created at the end of the homogenization (Argov et al., 2008). Although the original MFGM remains on the fat globules (Keenan et al., 1983), it is inadequate to cover the newly formed fat surface (Sharma and Dalgleish, 1993). As such, the surface active proteins, especially caseins, cover the newly formed surface of the fat globules. This protects the fat from coalescence. This process changes the milk fat globule unique macrostructure since the newly formed fat globules exhibit different physical and chemical properties (Argov et al., 2008). Heating of homogenized milk changes both physical and chemical properties in MFGM and milk proteins due to their interaction with each other (Van Boekel and Walstra, 1989; Sharma and Dalgleish, 1993). Homogenization must occur at temperature 55-80°C since milk fat must be liquid and native milk lipase must be
inactivated. The presence of a rancid off-flavor in homogenized milk is an indication that either (1) all the milk ingredients were not adequately heat-treated prior to homogenization or (2) rancidity existed within the milk prior to the homogenization process (Alvarez, 2009). Homogenization at temperature below 40°C causes the partial solidification of milk fat results in incomplete dispersion of the fat phase.

Lipids are divided into three classes: (1) neutral lipids, which are esters of glycerol and fatty acids, (2) polar lipids, containing phosphoric acid, a nitrogen-containing compound, or oligosaccharide, and (3) miscellaneous lipids, a heterogeneous group of compounds including cholesterol, carotenoids, and fat-soluble vitamins (Fox, 2009). Milk fat is composed of 95-98% of TG, and the remaining constituents are phospholipids, cholesterol, diglycerides, monoglycerides, and free fatty acids (Bauman and Griinari, 2001; Jensen et al., 1991; MacGibbon and Taylor, 2006). Milk fat contains approximately 400 fatty acids, although most of these are present at trace levels (Fox, 2009). The major fatty acids of milk TG include straight chain fatty acids that are saturated and have 4 to 18 carbons (4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 16:0, 18:0), monounsaturated fatty acids (MUFAs; 16:1, 18:1), and polyunsaturated fatty acids (PUFAs; 18:2, 18:3; Table 1.1) either in their free state or bound as glycerides (Jensen et al., 1991). The fatty acids of bovine milk fat originate from two sources; (1) synthesis in the mammary glands from acetate and 3-hydroxybutyrate, which generates C4 to C14 and some of C16, and (2) the uptake of preformed long chain fatty acids from the feed and diet, which consists of a portion of C16 and C18 fatty acids (MacGibbon and Taylor, 2006; Bauman and Griinari, 2001). Cis-PUFAs, specifically 18:2 and 18:3, also known as essential fatty acids, originate from the diet as cows cannot synthesize these physiologically (MacGibbon and Taylor, 2006). Milk fat contains low levels of PUFAs because PUFAs in the diet are hydrogenated by
bacteria in the rumen (Fox, 2009). Incomplete biohydrogenation by the rumen bacterium results
in the formation of conjugated linoleic acid (CLA; Fox, 2009). Minor fatty acids, such as
branched-chain, odd-numbered carbon fatty acids, hydroxyl and keto (oxo) fatty acids are
present at levels > 0.01% in milk (MacGibbon and Taylor, 2006; Jensen, 2002). Although these
are small amounts of hydroxyl and keto acids, they are important in milk flavor, contributing
lactones and methyl ketones, respectively (Fox, 2009).

The fatty acids are arranged on the TG molecule and esterified at three \( sn \)-positions
(Figure 1.1). These positions affect the physical, texture, and rheological properties of TG along
with fatty acid composition (Frankel, 2005). Most of the short chain fatty acids (SCFAs) are at
the bottom carbon position (\( sn \)-3) of the TG molecule whereas the longer fatty acids tend to be in
the middle (\( sn \)-2) and top positions (\( sn \)-1). The distinctive feature of bovine milk fat is the
presence of the SCFAs, 4:0 and 6:0, which are esterified almost exclusively at the \( sn \)-3 position
(MacGibbon and Taylor, 2006; Fox, 2009). The fatty acid composition of milk is not constant
throughout the cow's lactation cycle and feeding regimen, but it does not have a great impact on
the nutritional and physical properties (Smet et al., 2009) or the overall pattern of fatty acid
distribution (MacGibbon and Taylor, 2006). However, changes in composition may increase
spontaneous oxidation of milks, which can contribute to flavors. For example, the C14:0 and
C16:0 \( \beta \)-hydroxy fatty acids spontaneously form lactones upon heating which can enhance
buttery flavor. The location of the short- and medium-chain fatty acids (C4 to C10) in the
primary positions of TG makes them susceptible to lipases (Jensen, 2002). In particular, the
SCFA (C4 to C8) have very strong sensory characteristics with low flavor thresholds (parts per
million) and impart distinctive flavors in dairy products. The proportion of 18:2 and 18:3 appear
to be affected by the cow’s diet (MacGibbon and Taylor, 2006), which further generates different flavor compounds (Table 1.1).

Proteins

Milk proteins are the most characterized of all food proteins with unique properties (Fox, 2009). The two major categories of milk protein, casein and whey, are defined by their chemical composition and physical properties. Approximately, 82% of milk protein is casein and the remaining 18% is serum or whey protein. The casein family is in the form of micelles as large aggregates of phosphoproteins held together by calcium bridges and hydrophobic interaction. It is dispersed in milk and coagulates or precipitates at pH 4.6. Therefore, the term casein is for the insoluble protein at pH 4.6 in milk, and it is easily separated from milk, either by acid precipitation or by adding rennin (Fox, 2009). The whey protein remains in milk at pH 4.6. When the fat and casein have been removed from milk, one is left with whey (serum) proteins, which remain in milk at pH 4.6. The whey proteins in milk are small quaternary structures and made up of a number of distinct proteins, especially β-lactoglobulin (β-lg) and α-lactalbumin (α-la) (Figure 1.2). β-Lg accounts for about 50% of the whey proteins and has a high content of essential amino acids. They denature and form a complex with κ-casein when milk is heated to more than 75°C. The functional properties of milk are impacted by whey protein denaturation, most notably increase in viscosity (Lee et al., 2017).

Milk proteins are denatured by heat treatment (temperature), which involves unfolding and subsequent aggregation of unfolded protein molecules and flavor deterioration (Kühn et al., 2006). The casein micelle is stable to heat, and good quality milk can withstand up to 140°C for at least 10 min, often without coagulating (Lewis and Deeth, 2009). As such, casein permits
relatively small physical changes due to the lack of stable secondary and tertiary structures (Fox, 2009). However, whey proteins are completely denatured by heating at 90°C for 10 min (Fox, 2009).

**Enzymes**

Approximately 70 indigenous enzymes have been reported in normal bovine milk (Fox and Kelly, 2006). The indigenous enzymes are secreted and can arise from four principal sources: (1) blood plasma between mammary cells, (2) secretory cell cytoplasm, which may be entrapped within MFGM during excretion from the cell, (3) the MFGM itself, or (4) somatic cells, which enter the mammary gland from the blood to fight bacterial infection (Fox and Kelly, 2006). Major enzymes, such as plasmin, lipoprotein lipase (LPL), alkaline phosphatase (ALP), sulphydryl oxidase, and lactoperoxidase (LPO) have a potential significance in milk quality from the following viewpoints: deterioration (e.g., LPL), preservation (e.g., LPO and sulphydryl oxidase), thermal indicator (e.g., ALP, LPO and catalase), mastitis infection indicator (e.g., catalase and acid phosphatase) and antimicrobial activity (e.g., lysozyme and LPO) (Swaisgood, 1995; Fox and Kelly, 2006). LPL, potentially the most significant enzyme in milk, is also largely associated with casein micelles assisting initial digestion and absorption of milk lipids. However, its activity can cause flavor deterioration by hydrolytic rancidity (Swaisgood, 1995; Fox and Kelly, 2006). The activity of LPO has been used to prevent microbial deterioration (Swaisgood, 1995). Plasmin is associated with stability of casein micelles in ultra-high treatment (UHT) milk (Swaisgood, 1995). ALP is an indicator of adequate pasteurization, and sulphydryl oxidase catalyzes the oxidation of thiols and disulfide bonds (Swaisgood, 1995). The majority of native enzymes are associated with the MFGM complex. The other enzymes are dispersed in the serum.
phase or bound to the casein micelles (Walstra et al., 1999). Since there are no nutritional benefits from indigenous enzymes, destruction of these enzymes by heat is one of the objectives of many dairy processing (Fox and Kelly, 2006).

Some indigenous enzymes have significant effects on milk flavor. The most important of these are lipases and proteinases (Chen et al., 2003). Two types of lipolytic enzymes have a significant role in rancid and fruity flavors in milk: native, excreted by the mammary gland, and microbial, secreted by microorganisms or released after lysis (Walstra et al., 1999). LPL is the major native enzyme in milk, which is synthesized in mammary gland secretory cells (Chen et al., 2003). Typically, fresh bovine milk contains 0.5-2.0 mg/L of LPL. The level of LPL in milk is dependent on the breed, stage of lactation, season, etc. (Deeth and Fitzgerald, 1976). LPL is not normally active against milk fat in fresh milk because milk fat globules are protected by the MFGM (Chen et al., 2003). Although the MFGM can prevent LPL from coming into contact with milk fat, external stimuli such as agitation, and homogenization disrupt the MFGM and damage to the MFGM can initiate lipolysis (Deeth, 2006). The harsher the physical treatment or the longer time the treatment is applied, the greater the exposed surface area of the lipid, and the greater the rancidity (Campbell and Drake, 2013). Lipolysis only occurs when LPL directly contacts with milk fat and only when the MFGM is physically damaged (Fox, 2003). In addition, milk has enough natural LPL that can cause raw milk to become unacceptably rancid in less than 10 min if the MFGM is damaged (Deeth, 2006). Raw milk is not exposed to excessive agitation for this reason (Campbell and Drake, 2013). LPL is heat sensitive and destroyed by high temperature short time (HTST) pasteurization (Campbell and Drake, 2013). However, if not all LPL is inactivated by pasteurization, rancid flavor can develop after a period of storage (Deeth, 2006). While LPL is associated with fresh raw milk, microbial lipase is related to contamination
from the interior of the cow’s udder and teats, milking and/or storage equipment. (Collins et al., 2003; Chen et al., 2003). The contaminants are almost entirely psychrotrophs, mainly *Pseudomonas* species (Chen et al., 2003). *Pseudomonas* species are responsible for microbial lipases or extracellular phospholipases in milk (Deeth and Fitz Gerald, 2006). Phospholipases are potentially important in milk because of their ability to degrade the phospholipids of the MFGM, thereby increasing the susceptibility of the milk fat to lipolytic attack (Deeth and Fitz Gerald, 2006). Psychrotrophic *Pseudomonas* species in milk do not survive heat treatment such as HTST (Chen et al., 2003).

Lipolytic activity leads to the increase in free fatty acid (FFA) levels and rancid flavor in milk, which is generally unacceptable to consumers (Santos et al., 2003a; Mannion et al., 2016). FFA is a liberated form as a fatty acid is not bound to glycerol (Deeth and Fitz Gerald, 2006), especially when glycerides breakdown by either indigenous LPL present in raw milk or bacterial lipases in pasteurized milk (Antonelli et al., 2002). LPL is responsible for spontaneous lipolysis, which leads to the formation of FFA in fresh milk (Olivecrona et al., 1992). FFA profile can be varied because lipases have specificity towards substrate features, such as the primary position of TG and fatty acid chain length preference (Jensen et al., 1983). For example, LPL is 1,3-specific on TG, but the rate of hydrolysis of the sn-1 position is slightly faster than that of the sn-3 position (Olivecrona and Bengtsson, 1984). Lipase from ruminant sources tend to prefer butanoic acid from TG, but *Pseudomonas* sources are less relevant to SCFAs. In general, short chain FFA (< 12 carbons) has a greater impact on flavor changes than longer chain because the lipase preferentially hydrolyzes C4 to C12 more rapidly than the longer chain (Jensen, 2002). FFA flavors caused by short chains are described as vinegar, cheesy, sweaty, and soapy (Drake et al., 2001). Butanoic acid is especially associated with strong rancid odor (Mannion et al.,
Lipolytic associated aromas can be different from Pseudomonas strains (Stead, 1987; Hayes et al., 2002). Lipase from *Pseudomonas fragi* can cause fruity flavor by esterification of hydrolyzed milk fat and ethanol (Morgan, 1976). *Pseudomonas putida* produce fruity, cheesy, rotten and barn aromas (Hayes et al., 2002).

Proteolytic activity can result in the accumulation of small peptides causing development of bitterness (Ma et al., 2000), and astringency (Harwalkar et al., 1993). It is also associated with unclean and putrid flavors (Fairbairn and Law, 1986). As an indigenous enzyme, plasmin is responsible for most of the proteolysis in milk, and mainly acts on casein (de Rham and Andrews, 1982; Santos et al., 2003a). Mastitis can increase plasmin activity in milk (Chen et al., 2003). This corresponds to high levels of milk somatic cell count (SCC). On the other hand, bacterial proteinases from *Pseudomonas* and *Bacillus* species are the main source of spoilage in stored milk (Chen et al., 2003; Campbell and Drake, 2013). Proteinase production from psychrotrophs is normally maximum at the late log and early stationary phases of growth (Deeth and Fitz Gerald, 2006). *Pseudomonas* preferentially attacks casein over whey proteins, further affecting milk quality (Chen et al., 2003). In contrast, *Bacillus* species can be introduced into milk during production, and processing. As *Bacillus* species are heat-resistant and their spores can withstand thermal processing, they have been used as criteria for UHT milk quality (Chen et al., 2003; Hammer et al., 1995). Generally, *Bacillus* species have more diverse proteolytic activity than *Pseudomonas* and may produce more than one type of proteinase (Chen et al., 2003).

Measurement of enzyme activity has received considerable attention because of the defects caused in stored milk and dairy products, (Deeth and Touch, 2000; Chen et al., 2003). Santos et al. (2003a) which demonstrated that measurement of lipolysis and proteolysis can
provide chemical benchmarks of off-flavors caused by native enzyme activity in milk. Moreover, the indigenous milk enzymes have no beneficial effect on the nutritional attributes of milk. Therefore, their destruction by heat is one of the objectives of dairy processing (Fox and Kelly, 2006). Inactivation of enzymes greatly impacts milk quality. This is due to possible quality failure by enzymatic spoilage and growth of bacteria. Inactivation is mostly achieved by heat treatment, which denatures enzymes because they are proteins (Walstra et al., 1999). Heating time-temperature may differ from various enzymes due to differences in heat stability (Walstra et al., 1999; Table 2). Most enzymes in milk are inactivated by 75°C (Lewis and Deeth, 2009).

**Carbohydrates**

Lactose is the major carbohydrate fraction in milk. It consists of two sugars, glucose and galactose (Figure 1.3). Lactose is mainly dissolved in the serum phase of fluid milk. Most of the free monosaccharides in milk are galactose and glucose (Adachi and Patton, 1961). Lactulose, known as a secondarily formed carbohydrate in milk products, is produced from molecular rearrangement of lactose during heat processing and storage (Adachi and Patton, 1961). Lactulose is formed in heated milk products by two possible mechanisms: an isomerization of lactose under mainly alkaline condition, and Amadori rearrangement by hydrolytic degradation (Adachi and Patton, 1961). Interest in lactulose in terms of quality has increased in recent years because it is highly soluble in water, has a sweeter taste than lactose and does not crystallize easily (Adachi and Patton, 1961).

The normal pasteurization conditions used for fluid milk have no significant effect on lactose. The higher temperatures used for ultrapasteurization (UP) or UHT of ESL milks and
spray drying can cause browning and isomerization reactions, which may affect product quality and nutritional properties.

**Flavor Formation of Milk**

Raw milk quality influences shelf life and flavor quality of pasteurized fluid milk (Barbano et al., 2006). Raw milk flavor greatly impacts on the quality of various dairy products, including pasteurized milk and cheeses. Raw milk flavor is different from milk treated by heat, primarily due to the lack of heat-induced interactions of milk proteins. Two major sources that can influence flavor and quality of raw milk can be discussed: (1) microbial sources, and (2) feed constituents and bovine metabolism during milk formation.

**Influence of Microbial Source**

The Pasteurized Milk Ordinance (PMO) requires a minimum cooling temperature of 7°C for raw milk, and the total bacterial count in Grade A milk is < 100,000 cfu/mL for individual producer and 300,000 cfu/mL for commingled milk. Psychrotrophic bacteria can grow at 7°C and lower, and they dominate the flora during cold storage after milk collection (Hantsis-Zacharov and Halpern, 2007). Instant cooling of raw milk can slow bacterial growth (Guul-Simonsen et al., 1996), but it can also stimulate high level of FFA by pumping, which disrupts the MFGM allowing lipolysis to occur (Dickow et al., 2011). Their extracellular enzymes, mainly proteases and lipases, contribute to the spoilage of milk (Hantsis-Zacharov and Halpern, 2007). Generally, high levels of psychrotrophic bacteria in raw milk are required to contribute sufficient quantities of heat-stable proteases and lipases to cause breakdown of protein and fat after pasteurization (Barbano et al., 2006). When starting with raw milk that has a low bacterial
count and in the absence of microbial growth in pasteurized milk, enzymes associated with high
SCC will cause protein and fat degradation during refrigerated storage and produce off-flavors,
because increased SCC is correlated with increased amounts of heat-stable protease (plasmin)
and lipase (LPL) in milk (Barbano et al., 2006). Psychrotrophic gram-negative bacteria produce
heat-stable proteases and lipases, and a high level of these organisms in raw milk could
contribute heat-stable enzymes that may produce off-flavor issues later during the shelf life of
pasteurized milk (Barbano et al., 2006). In particular, *Pseudomonas* species would need to grow
to relatively high numbers (e.g., 1\times10^6) in raw milk before pasteurization to produce an off-
flavor directly (Barbano et al., 2006). However, if the raw milk used for fluid milk processing
conforms to the Grade A total bacteria count standards in the PMO, then psychrotrophic bacteria
count in raw milk should not be a problem in a good quality milk supply today (Barbano et al.,
2006).

Milk SCC is a commonly used indicator of the prevalence of mastitis in dairy cows,
which is a bacterial infection in a cow’s udder (National Mastitis Council, 1996). Milk from
infected cows is characterized by high levels of raw milk SCC (Ma et al., 2000). The PMO
defines SCC of Grade A raw milk as 750,000 cells/mL Most of the major and minor components
in milk are affected by mastitis (Munro et al., 1984). Using high SCC milk causes increased
proteolytic and lipolytic activity affecting sensory quality and shelf life of pasteurized milk
(Munro et al., 1984; Ma et al., 2000; Santos et al., 2003b), and lower cheese yield (Politis and
Ng-Kwai-Hang, 1988; Barbano et al., 1991; Klei et al., 1998). Studies have shown that mastitis
has an adverse effect on the shelf life and sensory quality of pasteurized milk, due to higher
concentration of FFA (Rogers and Mitchell, 1989), and rancidity and bitterness (Ma et al., 2000).
Cow Feeds and Bovine Metabolism

High quality milk can be achieved from good quality raw milk from the farm environment (Lewis and Deeth, 2009). Undesirable flavors in raw milk associated with the feeding of ensiled forages have been a problem in the dairy industry for a long time (Mounchili et al., 2005). The cow’s diet has a significant effect on the flavor of the dairy product and involves a high degree of lipolysis, which can release volatile fatty acids, methyl ketones, and lactones (Urbach, 1993). Occasionally, flavors transferred from interactions between the cow, its environment (mainly feed) and cow metabolism (e.g., respiratory, digestive system, and bloodstream) can remain in the final products. These flavors are commonly called transmitted flavors or taints, which are described as feed, weed, cowy, and barny flavors (Bassette et al., 1986; Jeon, 1996). Their contribution to fresh milk flavor were previously reported (Cadwallader and Howard, 1998). Transmitted flavors are defects caused by the transfer of aromatic substances either from the cows’ feed or their surroundings through the respiratory or digestive systems (metabolism) to the bloodstream and subsequently into the milk (Bassette et al., 1986; Shipe et al., 1978; Jeon, 1996). The mechanism of the flavor transmission appears to occur both through lungs to the udder and rumen walls to the bloodstream (Shipe et al., 1962). Volatile compounds are assumed to originate from not only the feed, but also the cow’s metabolism or digestion system (Jeon, 1996).

Cow feed has two main impacts on fatty acid composition linked to feed/weed flavors (Jeon, 1996) and lipid oxidation (Smet et al., 2009). Feed flavor is an undesirable flavor in fresh milk (Bassette et al., 1986), and can taint the milk if cows consume feed shortly before milking (Jeon, 1996; Mounchili et al., 2005). Feed flavor is commonly related to the type of feed: silage and green forage especially when the feed is changed from dry to lush forage (Shipe et al., 1978).
It is different from flavors resulting from decomposition of the milk by microorganisms, processing, or chemical changes during storage (Bassette et al., 1986). Probable markers for feed flavor are limonene (Jeon, 1996), ethanol (Shipe et al., 1962; Randby et al., 1998; Mounchili et al., 2005), acetone, 2-butanone, and dimethyl sulfide (Mounchili et al., 2005). Terpenes (limonene) are abundant in plant families (Abilleira et al., 2009), rapidly transferred from cow feeds (e.g., herbs or forages) into milk fat (Contarini et al., 1997; Viallon et al., 2000). Ethanol can be derived from fermented silages (Shipe et al., 1962; Randby et al., 1998). Ketones such as acetone and 2-butanone are imparted from cows fed silage shortly before milking (Calvo and Hoz, 1992; Mounchili et al., 2005). Increased acetone in milk is also associated with protein deficiency in feed when the cow suffers from ketosis (Walstra et al., 1999).

Cows often ingest enough weeds to produce an off-flavor or taint in the milk (Bassette et al., 1986). Weed flavor is a readily recognized off-flavor and described as garlic/onion flavor (Bassette et al., 1986). Skatole and indole have a strong unpleasant aroma, and can be produced from tryptophan in the cow’s rumen (Bendall, 2001). They are related to certain types of plants/weeds consumed by the cow (Bassette et al., 1986). Since tryptophan cannot be synthesized in the rumen, pasture-fed cows will have more free tryptophan than supplement-fed cows, and therefore more skatole and indole in their milk (Bendall, 2001). This circumstance can be applied to phenolic compound formation from tyrosine (Lane and Fraser, 1999). Cumarin is generated from hay feeding (Walstra et al., 1999). Barn and unclean odors may remain in milk because of poor ventilation or hygiene management (Bassette et al., 1986), but those flavors are uncommon in recent years due to the improved environment of dairy farms (Jeon, 1996).

Some cows produce milk with spontaneous oxidized flavor(s), such as fishy or metallic, which is developed without the addition of exogenous oxidants or exposure to light (Timmons et
Spontaneous oxidation of raw milk is mainly due to autoxidation of PUFA and imparted from various factors, such as feed quality, late lactation, mastitis, and the presence of antioxidants (Timmons et al., 2001; Alvarez, 2009). Feed quality can be determined by season, dietary supplement, or the type of feed (e.g. grass, silage), which affect fatty acid composition and the oxidative stability of milk (Barrefors et al., 1995). The fatty acid composition in milk, especially the linolenic acid (C18:3), is the most important for autoxidation susceptibility and is directly influenced by the type of feed (Havemose et al., 2007). Since most fatty acids stored in ruminant adipose tissue are saturated (C4:0 to C16:0), concentration of PUFA in milk is closely related to the diets and quantities absorbed in the rumen intestine (Chilliard and Ferlay, 2004). As such, supplementation with C18 fatty acids may affect autoxidation susceptibility of milk. Barrefors et al. (1995) noted that raw milk with higher concentrations of C18:2 and C18:3 had oxidized flavor, indicating that the proportions of C18:2 and C18:3 are important for the oxidative stability of milk. Increased linolenic acid is induced by pasture-fed because fresh green grass is the main source of C18:3, which explains why milk from grass-fed cows have more C18:3 than maize-based or concentrate-rich ones (Chilliard et al., 2001). Bendall (2001) demonstrated that C18:3 oxidation compounds, such as cis-1,5-octadien-3-one, cis-4-heptenal, and cis-3-hexenal were more important for fresh milk flavor than compounds from C18:2 or C18:1. As such, the level of C18:3 can be significant in fresh milk due to faster rates and shorter induction of autoxidation (Bendall, 2001). Linolenic acid concentration is highest in the spring and fall (Bauchart et al., 1984). In contrast, hay making considerably reduces linolenic acid concentration in forage (Boufaied et al., 2003).

Raw milk contains several non-enzymatic antioxidants (vitamin E, carotenoids, and vitamin C) that are important for the oxidative stability (Barrefors et al., 1995). α-Tocopherol is
located in the MFGM, and β-carotene is located in the neutral fat (Barrefors et al., 1995).

Alvarez (2009) demonstrated that the oxidation susceptibility of raw milk is more a function of presence/absence of natural antioxidants in pasture or green feeds, than the relative changes in levels of unsaturated fatty acids by milkfat composition within season. Barrefors et al. (1995) also noted the important role of α-tocopherol and β-carotene in the spontaneous oxidation of milk fat. α-Tocopherol protects the membranes from free radicals, and β-carotene is a scavenger of radicals and singlet oxygen which inhibits initial peroxide radicals (Barrefors et al., 1995). These antioxidants prevent the formation of hydroperoxides, which can further breakdown to volatile compounds, particularly 2-enals from C8 and C9 (Barrefors et al., 1995). Milk produced from cows on pastures is normally resistant to oxidation, because antioxidants are practically nonexistent in dried feed and especially low in alfalfa hay (winter feeding) (Alvarez, 2009). Thus, oxidation is usually greater in the winter months when pasture or green feeds are not available (Alvarez, 2009). The presence of antioxidants from pasture and/or green feeds in the spring through mid-fall seasons is important (Alvarez, 2009).

Changes in Milk Flavor in Processing and Post Processing

Thermal Processing of Milk

Pasteurization is a thermal treatment that provides acceptable microbial safety, shelf life, and flavor characteristics using a different range of conditions to reduce the number of spoilage bacteria and to eliminate pathogenic bacteria (Trujillo et al., 2002; Pereda et al., 2008). Heating types vary from low to high or ultra-high, but the main purpose is to reduce bacterial spoilage and eliminate any potential pathogenic microorganisms (Pereda et al., 2007; Lewis and Deeth, 2009). Pasteurization is a relatively mild heat treatment, resulting in products with a limited shelf
life at refrigerated conditions (Lewis, 1994). Additional heat treatment would enhance the shelf life for products designed to be stable at ambient temperatures, but it also affects the sensory properties of milk (Ochi et al., 2010). In general, pasteurized, UP and UHT processing are known for thermal treatments applied to milk, and are categorized regarding the thermal processing method, temperatures and time in accordance with the PMO.

The original method for pasteurizing milk was vat pasteurization or batch process, which heats milk in a large tank for at least 30 min at 63°C. It is still used in some small-scale processes (Lewis and Deeth, 2009), but is now primarily used for making starter cultures in the processing of cheese and yogurt, and for pasteurizing ice cream mixes. A continuous process is the most predominant in the U.S. (Lewis and Deeth, 2009). High temperature short time (HTST) pasteurization uses metal plates and hot water to raise milk temperatures to a minimum of 72°C for no less than 15 s, followed by rapid cooling. Another method, higher heat shorter time (HHST) is a process similar to HTST pasteurization, but it uses slightly different equipment and higher temperatures for a shorter time (Alvarez, 2009). UP or UHT can be used for ESL milk, which was introduced to protect both shelf life and temperature abuse (Lewis and Deeth, 2009; Colahan-Senderstrom and Peterson, 2005; Caplan and Barbano, 2013; Lee et al., 2017). UP milk must be heated to no less than 138°C for at least 2 s, and it allows a longer shelf life than HTST milk. However, UP milk still requires refrigerated distribution and storage because it is not shelf stable. UHT involves the same thermal process as UP milk, but the milk is shelf stable for up to 6 mo at ambient temperature due to aseptic filling. The basis of UHT aseptic packaging is sterilization of the product before packaging, then packaging in pre-sterilized containers in a closed sterile atmosphere. Thus, UHT milk needs no refrigeration until it is opened. UHT milk is more common in Europe due to the centralization of dairy industry, competition among dairy
companies, less frequent shopping cycles, and inadequate cold chain distribution maintenance (Rysstad and Kolstad, 2006; Heyndrickx et al., 2010). However, UHT and/or UP milk has a distinct cooked flavor which has not been preferred by consumers in the U.S. (Lee et al., 2017). It also can have defects in color and vitamin content compared to pasteurized milk (Nusrten, 1997).

Continuous processing includes two types of heating methods to reach the desired temperature: (1) direct heating, which quickly heats the milk by direct steam injection, and (2) indirect heating, which heats the milk by tubular, scraped surface heat exchangers or plate-heat exchangers indirectly (Boelrijk and De Jong, 2003; Jelen, 1982). The direct steam injection method maximizes heat transfer because the steam has direct contact with the product, and a vacuum chamber subsequently removes the equivalent amount of water added to the milk from the condensed steam (Tetra Pak, 2003). In contrast, indirect heating is achieved by an indirect approach through a heating medium (Tetra Pak, 2003). Overall, an indirect heating system has a greater heat exposure than direct heating under equivalent temperature because heating and cooling rates are slower (Lewis and Deeth, 2009; Jelen, 1982). In contrast, direct heating is known for its very short holding time and gentle heating (Rysstad and Kolstad, 2006). Consequently, a more cooked flavor can be expected in milk heated by indirect system because of a higher amount of heat load in product (Jelen, 1982). Direct heating is considered to have less chemical degradation and flavor defects (Rysstad and Kolstad, 2006). UP milk receives a significantly higher thermal load than HTST milk as shown by thermal damage indicators, such as furosine and serum protein denaturation (Lee et al., 2017). Due to differences in heat time, temperature, and total heat load, the sensory profiles of DSI-UP, IND-UP and HTST milks are
all distinct (Lee et al., 2017; Jo et al., 2018). The flavor of pasteurized milk is derived from various reactions during thermal processing (Cadwallader and Singh, 2009).

**Effect of Heat Treatment on Milk Flavors**

The Maillard reaction. Milk flavor is the result of the chemical interaction of proteins, lipids, and carbohydrates, which are the precursors of aroma compounds (Alvarez, 2009). Key changes in the flavor of many dairy products have been associated with the Maillard reaction (MR) during thermal treatment (Van Boekel, 1998; Colahan-Sederstrom and Peterson, 2005). The MR, a reaction between carbonyls and proteins or amino acids, mainly reducing sugars and amines, is one of the dominant pathways to develop flavors in foods under non-enzymatic browning (Reineccius, 2006). It is very important to produce not only desirable flavors, but undesirable flavors as a result of unwanted MR during processing or storage (Reineccius, 2006). In milk, it occurs between the lactose and free amino groups or protein, which produce flavors and color, and decreases the available content of the amino acid lysine in milk protein. Milk is especially sensitive to thermally induced non-enzymatic browning reactions due to its relatively high concentrations of lactose and lysine-rich proteins (Morales et al., 1997). In particular, lysine residues in k-caseins (more active than in whey protein) can easily react with lactose, thus reducing sugar during heat treatment of milk (Van Boekel, 1998). Other carbohydrates, such as monosaccharides, oligosaccharides, and lactulose, present at low levels in milk products and can also affect the formation of MR products (Adachi and Patton, 1961).

The MR can be divided into three stages: early, advanced, and final stage (Van Boekel, 1998). In the early stage, the aldehyde group of lactose reacts with ε-amino group of lysine and
forms a Schiff’s base, and then converts to an Amadori product, lactosyllysine (galactose-fructose-lysine, biologically not available) (O’Brien and Morrissey, 1989) (Figures 1.5 and 1.6).

In the following advanced stage, the Amadori product is broken down into three different reaction products depending on the pH (acidic, neutral, and basic) (Van Boekel, 1998). Under pH less than 7, the amino group of lactosyllysine is protonated, and 1,2-enolization through 3-deoxyosulose leads to 5-hydroxymethylfurural (HMF), furfuryl alcohol, and pyrraline, especially when a reducing sugar is a hexose (Morales et al., 1997). Similarly, the Amadori product would turn into furfural if the reducing sugar is a pentose instead of a hexose. In the foods with neutral and pH above 7, the reaction product is dominant via 2,3-enolization pathway (Figure 1.7). In this route, the Amadori product transforms into a variety of different products, such as colorless reductones, 3-furanone, β-pyranone, cyclopentenone, galactosylisomalto, acetylpyrrole, fluorescent substances and dicarbonyl products, which further react with amino compounds producing aldehydes and intermediates. For milk and milk products, 2,3-enolization pathway is more dominant than 1,2-enolization. The most important consequence of the advanced stage is Strecker degradation, which is the reaction between dicarbonyls and free amino acids (Reineccius, 2006). The end products of the Strecker degradation are CO₂, an amine, and aldehydes of each deaminated and decarboxylated amino acid (Reineccius, 2006). Amino acids are converted to aldehydes, known as Strecker aldehydes, which are very reactive and contribute significant flavors to heated food (Reineccius, 2006). Strecker aldehydes are also a source of browning since they can condense with themselves, with sugar fragments, furfural, or other dehydration products (Morales and van Boekel, 1997). Amino acids are the precursor of Strecker aldehydes, thus the reaction of certain amino acids with reducing sugars can be important for flavor.
In the final stage of MR, melanoidins (brown pigments) and non-volatile compounds are produced (Reineccius, 2006). It is also expected that lysine is involved in melanoidin formation because lysine loss increases during the final stage (Van Boekel, 1998). The quality of pasteurized milk is countered by this series of reactions due to the formation of a dark color and off-flavors, and the loss of nutritional value, particularly lysine (Shamberger and Labuza, 2006).

A number of target analytes have been used to evaluate the extent of MR in milk. The challenges here are to find the markers associated with different MR stages, quantify target analytes, and estimate the state of the reaction (Aalaei et al., 2018). Estimation of available lysine is a classic and conventional measure of heat damage (Morales et al., 1995). Lysine loss is the main consequence in the initial stage of MR, but it can be quantified as free lysine when acid hydrolysis is applied (Erbersdobler and Somoza, 2007). It has provided a direct means of the early stage of the MR (Morales et al., 1995). However, further research revealed that available lysine is not the most sensitive marker of extensive heat treatment (Erbersdobler and Somoza, 2007). A variety of MR intermediates including HMF, furosine, N-carboxymethyllysine (CML), pyrraline, and pentosidine are widely used as markers for pasteurized milk or UHT milk (Erbersdobler and Somoza, 2007). HMF has been considered as a heating or process-control parameter, since Keeney and Bassette (1959) developed a colorimetric method of determining HMF in dairy products. There are two pathways in HMF formations when sugars are heated: (1) from the Amadori products in the MR through enolization in the presence of amino groups in the medium, and (2) from lactose degradation and isomerization reaction (Morales et al., 1997). Depending on the method, it can determine either free HMF or total HMF, which is free HMF plus potential HMF from other intermediates (Burton, 1984). Furosine is an artificial amino acid formed during acid hydrolysis of Amadori products, which are produced by ε-amino groups of
lysine with glucose, lactose and maltose (Finot et al., 1981; Erbersdobler and Hupe, 1991). Since furosine was first identified in 1966, it has been used as a reliable indicator of thermal damage of proteins, demonstrating the importance of the Amadori products and the early stage of MR (Erbersdobler and Somoza, 2007). However, furosine content does not always increase with the severity of heat treatment (Erbersdobler and Somoza, 2007). In more severely heated products, the Amadori products and furosine decrease again due to formation of MR intermediates and end products (Erbersdobler and Somoza, 2007). CML is a marker for glycated proteins (Erbersdobler and Somoza, 2007). CML can provide additional information on the protein damage in foods where furosine levels have already decreased (Erbersdobler and Somoza, 2007). Pyrraline is formed significantly later than furosine and appears to be a useful marker of the advanced and late MR (Erbersdobler and Somoza, 2007). Pentosidine is a derivative of arginine and has been quantified in foods as a marker of heat treatment (Erbersdobler and Somoza, 2007). CML, pyrraline, and pentosidine are known as advanced glycation end products (AGEs), which are the molecules suspected for potential health risks (Aalaei et al., 2018). Lysylpyrraline, another AGE molecule was detected in heated milk as a marker for advanced stage of MR (Van Boekel, 1998).

In the aspect of milk and milk products, furosine and HMF have a significant role in the analytical, quality and nutritional relevance, because they are strictly linked to MR of milk products, although they have been applied to honey, jams, and cereal-based products (Morales et al., 2009). Both compounds are significant indicators of damage during heat treatment of milk; MR cannot be obtained without a formation of HMF, and furosine is a direct marker of lysine reaction products. In addition, the correlation between HMF and furosine contents prove to be good in UHT milk (Erbersdobler and Somoza, 2007). For the detection of furosine, high-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) have
produced reliable and accurate results (Singla et al., 2018). Furosine analysis with HPLC is preferred the most because it is an extremely simple procedure (Singla et al., 2018). In general, samples need to undergo acid hydrolysis to prevent furosine conversion in N-ε-(carboxymethyl)lysine. Analysis of furosine with HPLC has been widely applied to different milk products, especially after a pure and stable standard became available in 1992 (Erbersdobler and Somoza, 2007). By furosine, blocked lysine in the foods can be calculated (Aalaei et al., 2018). The most common choice for HMF analysis is the spectrophotometric method (Singla et al., 2018). However, it is not reliable because it has low accuracy and sensitivity, as well as possible interference by non-HMF analytes having same wavelength area (Markowicz Bastos et al. 2012). HPLC has often been used for HMF detection due to its versatility (Singla et al., 2018). This technique requires preliminary extraction steps, and solid phase extraction is generally applied. HPLC strategy appears to be useful for HMF, but the detection limit of HMF is very low, and it is usually used in conjunction with spectrophotometers (Singla et al., 2018).

On the other hand, pigment and color change have been monitored in milk as an indicator of the advanced stage of MR (Morales and van Boekel, 1997; Pagliarini et al., 1990). One of the methods include measuring fluorescence as a marker for the advanced stage of the MR in milk model system (Morales and van Boekel, 1997).

MR develops flavors associated with cooked and sweet aromatics in milk, primarily under high heat (> 140°C) (Belitz et al., 2009). Cooked flavor is affected by amino acid composition due to MR being highly selective in the presence of specific amino acids to generate characteristic flavors. These amino acids include cysteine, lysine, methionine, leucine, isoleucine, valine, and tryptophan as flavor precursors (Reineccius, 2006; Belitz et al., 2009). Strecker aldehydes serve as indicators of the MR and can further be involved in additional
reactions (Reineccius, 2006). 2 and 3-methylbutanal are formed via Strecker degradation of valine and leucine, respectively. Previous studies have reported increased levels or accumulation of 2 and 3-methylbutanal in UHT milks due to heat severity (Contarini et al., 1997; Valero et al., 2001; Contarini and Povolo, 2002; Vazquez-Landaverde et al., 2005; Belitz et al., 2009). 2-acetyl-1-pyrroline is formed in Strecker degradation of proline and readily forms even under mild heating (Reineccius, 2006; Belitz et al., 2009). Benzaldehyde, found in heated and UHT milk (Calvo and Hoz, 1992), may be generated via phenylalanine residue in casein with lactose (Jeon et al., 1978). Degradation of tryptophan by heat and alkali conditions is the primary source of 2-aminoacetophenone in milk proteins. Aminoacetophenone can be formed from the oxidation of skatole, which is derived from tryptophan (Belitz et al., 2009). Since tryptophan content is higher in the serum protein fraction than casein (Belitz et al., 2009), the formation of 2-aminoacetophenone could be more related to serum denaturation and would be expected to be more prevalent in UP milks. Aminoacetophenone is generally labeled as a stale or gluey off-flavor (Reineccius, 2006), but has also been attributed to grape and tortilla aromas in milk proteins and powders (Smith et al., 2016).

Aldehydes derived from sulfur-containing amino acids (cysteine and methionine) degrade further to volatile sulfur compounds, such as hydrogen sulfide, and methanethiol (Arnoldi, 2003). Strecker degradation of methionine has been suggested to form methional (Figure 1.8) followed by a formation of methanethiol, which is readily oxidized to dimethyl disulfide and dimethyl sulfide (Al-Attabi et al., 2009; Belitz et al., 2009). Methional was reported as a major off-flavor marker of UHT milk (Colahan-Senderstrom and Peterson, 2005; Troise et al., 2014). Methanethiol is known for its role in distinctive sulfur aroma, and methionine is generally accepted as the most important precursor for methianethiol via MR (Lindsay and Rippe, 1986).
However, it is extremely volatile and readily oxidative in the presence of oxygen to yield dimethyl disulfide (Lindsay and Rippe, 1986). Dimethyl trisulfide is subsequently generated via oxidation of dimethyl sulfide where dimethyl sulfoxide is formed as an intermediate compound (Al-Attabi et al., 2009). Dimethyl sulfoxide is formed as an intermediate compound from the oxidation of dimethyl sulfide (Shibamoto et al., 1980), but its contribution to milk flavor is not confirmed yet. Among the numerous volatile sulfur compounds, thiazoles and thiophenes are major sulfur-containing compounds generated via MR (Reineccius, 2006). These are formed via the reaction of sulfur-containing amino acids with intermediates of MR (Reineccius, 2006).

Negative aspects of the MR during milk processing or storage receive attention than other off-flavor reactions, such as lipid oxidation. The flavor differences between pasteurization and UHT treatment have a significant relation to MR (Arnoldi et al., 2003). According to Valero et al. (2000), UHT milk had a negative impact on flavor changes mainly due to newly formed compounds from MR and proteolysis. Similarly, Jo et al. (2018) recently reported that increases in MR compounds were more prevalent in milk by UP-indirect heating (IND-UP) than by UP-direct steam injection (DSI-UP) or HTST due to the greater heating load of indirect heating. The key compounds included 2 and 3 methylbutanal, furfural, 2-acetyl-1-pyrroline, 2-aminoacetophenone, and benzaldehyde (Jo et al., 2018). On the other hand, compounds derived from sugar degradation during MR were predominant and are likely to increase sweet aromatic flavors in UP milks (Jo et al., 2018). Lactose and its hydrolysis products, glucose and galactose can produce aroma compounds during thermal processing. Furfural is derived from sugars during heat processing, and it was previously reported as an indicator of heat treatment and a key compound of UP milk (Vazquez-Landaverde et al., 2005; Colahan-Senderstrom and Peterson, 2005). Furfural is also known as the precursor of melanoidins in MR in milk, which contribute to
its darker color (BeMiller and Whistler, 1996). Maltol and diacetyl are derived from lactose via nonenzymatic browning reaction, especially in heated milk (Calvo and Hoz, 1992). Diacetyl is especially known as an important contributor to the buttery note in heated milk (Scanlan et al., 1968).

**Protein denaturation.** Casein and β-lg in serum protein, the primary proteins found in milk, are associated with flavor formation and heat denaturation during processing. The key aspect of milk proteins as flavor precursors is amino acid composition. The casein contains a high level of proline, and is low in sulfur amino acids (Fox, 2009). The sulfur amino acid in casein is mainly methionine, with little cystine or cysteine. In contrast, serum proteins are relatively rich in cystine and/or cysteine.

Sulfur compounds have been associated with undesirable off-flavors, such as intense cooked and sulfur/eggy flavors in UP/UHT milk. Although sulfur compounds are naturally present in raw milk (Al-Attabi et al., 2009), these compounds are generated during thermal denaturation of serum proteins (Vazquez-Landaverde et al., 2006). The impact of sulfur compounds on milk flavor was predominant in UP milks compared to HTST (Jo et al., 2018). Jo et al. (2018) reported hydrogen sulfide, carbon disulfide, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, and methional as key sulfur compounds in UP milks. Among these, hydrogen sulfide and carbon disulfide were responsible for higher sulfur/eggy flavor and cooked flavor in UP milks, respectively. The primary source of sulfur compounds in milk can be generated by two reactions: Strecker degradation of sulfur amino acids (cysteine/cystine, and methionine), and the liberation of sulfur compounds by sulfur containing amino acids (cysteine) in β-lg serum protein denaturation (Mehta, 2015). When free reactive sulfides are denatured by heat (Calvo
and Hoz, 1992), sulfur compounds can be liberated as hydrogen sulfide, carbonyl sulfide, methanethiol, methional, dimethyl sulfide, dimethyl sulfone, and carbon disulfide leading to a cooked/cabbage flavor (Calvo and Hoz, 1992; Vazquez-Landaverde et al., 2006). Formation of hydrogen sulfide has been suggested in various ways: thermal denaturation of β-lg (Hutton and Patto, 1952), Strecker degradation of cysteine and methionine (Belitz et al., 2009), and thermal degradation of thiamine (Dwivedi and Arnold, 1973). The denaturation of individual serum proteins has been hypothesized as a primary source of hydrogen sulfide in milk because serum proteins are more sensitive to heat than casein (Mehta, 2015). The β-lg in serum protein contains five cysteine residues capable of forming disulfide bonds leading to further flavor development (Mehta, 2015). When β-lg is denatured, the sulfhydryl groups could readily release hydrogen sulfide and subsequently form disulfide bonds or further react with other milk proteins (Mehta, 2015).

**Lipid Degradation.** Lipid degradation during thermal processing generates methyl ketones, lactones, and aldehydes (Calvo and Hoz, 1992). Methyl ketones are naturally present in milk, but their formation can be enhanced by β-oxidation of saturated fatty acids followed by decarboxylation of β-keto acids (Grosch, 1982; Calvo and Hoz, 1992; Nursten, 1997). Milk contains 1% lipids as oxo fatty acids, which can be liberated as β-ketoacids and decarboxylated to methyl ketones of C6-16 when the milk fat is heated in the presence of water (Grosch, 1982). Their concentration increases during severe thermal temperature (Vazquez-Landaverde et al., 2005) or storage (Contarini et al., 1997; Valero et al., 2001). Overall, methyl ketones (e.g., 2-heptanone, 2-nonanone, and 2-pentanone) were more correlated with perceived stale flavor than with cooked flavor when concentrations increased in UHT milk (Contraini and Povolo, 2002).
Lactones have been traditionally associated with milk products (Maga, 1976), and make a significant contribution to the flavor (Reineccius, 2006). Lactones can be formed via various routes, including microbial, thermal, oxidative, and enzymatic (Maga, 1976). In milk, lactones are formed via hydroxy acids, which are relatively unstable and readily hydrolyzed from TG in the presence of water with heat (Reineccius, 2006). Hydroxy acids have no flavor as long as they are bound to TG, but heat treatment liberates acids and leads to hydrolysis of hydroxyl acids, which can then cycle to a lactone (Reineccius, 2006). Milk lipids from C7 to C16 have been related to lactone formation, and the levels can be affected by breeds, stage of lactation, and seasons (Maga, 1976), contributing characteristic milkfat, caramelized, and sweet flavors or coconut-like and peach-like flavors in milk (Reineccius, 2006; Maga, 1976; Drake et al., 2001).

*Changes of Milk Flavor during Storage*

The loss of characteristic flavor due to evaporation or compound reaction with the food itself is also considered an off-flavor (Reineccius, 2006). Many of the off-flavors present in fluid milk are more delicate, less volatile, or otherwise more elusive than those typically encountered in other dairy foods (Alvarez, 2009). The oxidation of lipids is among the most common cause of deterioration in food and mostly related to off-flavors. Rancidity can be a serious problem in many lipid rich foods including milk, causing undesirable rancid flavors and decreasing the nutritional value by the formation of secondary products (Frankel, 2005). Triglycerides in milk are susceptible to two types of deterioration: hydrolytic rancidity by enzymes and oxidative rancidity by chemical action (e.g., light exposure or oxidation) (Fox, 2009). Each of these processes proceeds through different mechanisms.
**Hydrolytic reaction.** Hydrolytic rancidity in milk results from the enzymatic hydrolysis of milk lipids described previously. This process is also known as lipolysis, which is catalyzed by lipases of milk lipids and produces FFAs and partial glycerides (Frankel, 2005; Deeth and Fitz Gerald, 2006). Several factors, such as type of enzymes, lactation stage of cow, and processing can be associated with the activation or inactivation of lipolysis. LPL is normally inactive because it is separated from the triglyceride substrates by the MFGM. However, if the membrane is damaged, lipolysis and hydrolytic rancidity occur rapidly (Fox, 2009). LPL mainly liberates short- and medium-chain fatty acids from lipoproteins and chylomicrons (Walstra et al., 1999; Fox, 2009). Raw milk contains a relatively large amount of lipase activity. It is readily initiated by vigorous agitation, which disrupts the milk fat globule membrane and renders triglycerides more accessible to milk (Deeth and Fitz Gerald, 2006). A low level of lipolysis is desirable in some cheeses for flavor formation, but hydrolytic rancidity by LPL is a potentially serious issue in raw milk and milk products (Fox, 2009). The flavors from hydrolytic rancidity are described as rancid, butyric, bitter, unclean, and soapy in milk (Walstra et al., 1999).

**Oxidative reaction.** The oxidation mechanism is when oxygen attacks double bonds in the unsaturated fatty acids producing free radicals. Free radicals are molecules with unpaired elections, making them very reactive (McClements and Decker, 2008), and they oxidize by causing hydrogen abstraction. The free radicals formed on unsaturated lipids form hydroperoxides and other oxygenated species. The formation of the initial free radicals is affected by several factors, such as metal complexes, active oxygen, light, and heat. Autoxidation refers to a complex sequence of chemical changes that come from the interaction of unsaturated lipids with oxygen under mild conditions (Frankel, 2005; McClements
and Decker, 2008). It is highly affected by temperature, oxygen, degree of fatty acid composition, and the activity of pro and anti-oxidants (McClements and Decker, 2008).

Autoxidation can be broken down into 3 steps: initiation, propagation, and termination. In the initiation step, an alkyl radical is formed and is stabilized by the delocalization of the radical over the double bonds (McClements and Decker, 2008). These fatty acid radicals are more easily formed with increasing unsaturation. The double bonds in unsaturated fatty acids require less energy for hydrogen abstraction than do saturated fatty acids. This explains why unsaturated fats oxidize at room temperature whereas saturated fats do not (McClements and Decker, 2008).

During propagation, oxygen covalently bonds to the fatty acid at the site of the radical because oxygen has 2 radicals. As a result, 1 radical is still left on the oxygen species, resulting in a peroxyl radical (LOO·) (McClements and Decker, 2008). This peroxyl radical can further abstract a hydrogen atom from another molecule forming the hydroperoxide (LOOH), propagating the radical to another molecule. In the final step, termination, two radical species react with each other to form a non-reactive molecule.

The hydroperoxide from autoxidation does not have flavor or taste itself, but it breaks down to generate an alkoxy free radical, which is linked to the formation of secondary oxidation products, such as aldehydes, and alcohols (Hamilton, 2003). Lipid oxidation generates straight chain aldehydes (Figure 1.9) (Nursten, 1997).

*Photooxidation of milk and oxidized flavor.* Fluid milk is commercially sold in display shelves or cases with light, both natural and artificial under refrigerated temperature. This light, particularly in wavelengths ranging from 420 to 520 nm, can induce quality defects in milk (Bosset et al., 1994). Exposure to near-UV and any visible light source, such as sunshine,
fluorescent light (FL), or light-emitting diodes (LED), causes photodegradation reactions of photosensitizers in milks, generating singlet oxygen (Brothersen et al., 2016). Singlet oxygen is a highly reactive, electrophilic, and non-radical molecule (Min and Boff, 2002). It is generated from triplet oxygen by photosensitized reactions in the presence of light and a sensitizer (Min and Boff, 2002). Photosensitizers including chlorophyll, riboflavin, and synthetic colorants in foods can absorb energy from light and transfer it to triplet oxygen to form singlet oxygen (Min and Boff, 2002). The singlet oxygen oxidation is very significant because (1) it can oxidize and degrade amino acids which have electron-rich sulfur atoms (Michaeli and Feitelson, 1994); (2) it can directly react with electron-rich double bonds of unsaturated fatty acids without the formation of free-radical intermediates (Min and Boff, 2002); (3) it further reacts with several key nutrients, for example, vitamin D, and ascorbic acid (Min and Boff, 2002; Cadwallader and Howard, 1998); (4) it rapidly increases even at very low temperature (Rawls and VanSanten, 1970); and (5) it generates new compounds, which are not found in ordinary triplet oxygen oxidation (Frankel et al., 1981). More importantly, the effect of photooxidation is faster than autoxidation because there is no lag (induction) phase (Hamilton, 2003).

Light-induced off-flavors involve changes in lipids and protein; typical lipid oxidation of unsaturated fatty acids, and protein and/or amino acids degradation that rapidly occurs (Reineccius, 2006). The key factor in light-induced oxidation of milk is riboflavin (Vitamin B2), which is present in large amounts in milk, as a water-soluble vitamin with an average concentration between 1.36 and 1.75 mg/L (Dimick 1982; Zygoura 2004). It is a strong photosensitizer, which has a triene structure and many double bonds that can further generate singlet oxygen under light (Bradley and Min, 1992; Mestdagh et al., 2005; Smet et al., 2009). Riboflavin easily absorbs light at wavelengths between 200 and 500 nm (Drösseler et al., 2002),
and excited riboflavin is able to generate highly reactive singlet oxygen or free radicals, which causes reactions with other compounds, such as amino acids or unsaturated fatty acids, and rapid loss of riboflavin by singlet oxygen oxidation (Dimick, 1982; Choe et al., 2005; Min and Boff, 2002). Excited riboflavin also generates superoxide anions, which can subsequently form singlet oxygen or other activated oxygen species that are able to react with lipid and initiate the oxidation of polyunsaturated fatty acids (Cadwallader and Howard, 1998; Skibsted, 2000; Walsh et al., 2015). It can be further associated with the formation of numerous volatile carbonyl compounds. The reaction rate of singlet oxygen with unsaturated fatty acids is relatively proportional to the number of double bonds in the molecules (Min and Boff, 2002). Singlet oxygen primarily reacts with 5 amino acids: tryptophan, histidine, tyrosine, methionine, and cysteine (Michaeli and Feitelson 1995 and 1997). Methionine and cysteine, rich in milk, have an electron rich sulfur atom, which can readily react with singlet oxygen (Min and Boff, 2002). In the presence of riboflavin and light, photoreduction of riboflavin forms methional via Strecker degradation of methionine (Cadwallader and Howard, 1998).

The extent of degradation and loss of milk nutrients by light exposure are dependent upon the type of light, light intensity, exposure time, packaging materials (Min and Boff, 2002; Whited et al., 2002; Cadwallader and Howard, 1998), and fat content of milk (Brothersen et al., 2016). Light exposure to 450 nm, which corresponds to the maximum absorption of riboflavin, is the most destructive to riboflavin (Min and Boff, 2002). Sunlight is more detrimental to riboflavin loss than FL (Min and Boff, 2002). FL results in greater changes in loss of riboflavin and vitamin A in milk compared to LED (Brothersen et al., 2016). The presence of milk fat appears to protect against vitamin A degradation (Whited et al., 2002). Higher fat content decreases photooxidation possibly due to decreased light penetration (deMan, 1981).
Light oxidized off-flavor in milk is generally determined by light induced oxidation reactions (Nursten, 1997; Marsili and Miller, 1998). Flavor changes can occur rapidly within 2 h under light exposure (Chapman et al., 2002), and increase during the storage of milk in refrigerated display cases and use of light transparent blow molded polyethylene containers for packaging milk (Hansen et al., 1975). Previous studies reported aldehydes, ketones, and volatile sulfur-compounds (Jung et al., 1998; Brothersen et al., 2016; Cadwallader and Howard, 1998) as light-induced flavor compounds, and these are related to singlet oxygen oxidation with fatty acids and amino acids. Jung et al. (1998) reported sulfurous off-flavor in milk exposed to sunlight for 15 min, and suggested dimethyl disulfide as a key compound derived from the reaction between singlet oxygen and methionine. Light can act as a pro-oxidant producing free radicals, which can react with unsaturated fatty acids in milk and further generate aldehydes and ketones (Jeon, 1996). Brothersen et al. (2016) noted that milk exposed to FL and LED had decreased cooked/sweet, and milkfat flavors and increased butterscotch and cardboard flavors, and astringency. Previous studies have also demonstrated light oxidized off-flavors are not appealing to consumers. Brothersen et al. (2016) reported that consumers detected off-flavors from milk exposed to FL and LED after 12 h and 24 h, respectively. Chapman et al. (2002) demonstrated that untrained consumers detected light-oxidized flavor defects between 54 min and 2 h of FL exposure of milks. Also, light exposed to 2% and skim milks received lower preference scores compared to non-exposed controls (Fellman et al., 1991).

**Effect of photooxidation on vitamin degradation in milk.** Vitamin fortification is a standard procedure for pasteurized fluid milks in the U.S. and vitamin concentrates are added to milk before pasteurization (PMO, 2015). Vitamin A fortification is also required in low fat (21 CFR
131.135) and nonfat milk (21 CFR 131.143), and vitamin D must be fortified with all fluid pasteurized milk according to the federal regulation. Vitamin A is typically fortified into reduced fat and skim milk at a minimum of 2000 International Units (IU) per quart, while vitamin D is fortified at a minimum level of 400 IU per quart (Yeh et al., 2017b). Vitamin A degradation by light exposure was reported in relation to off-flavor defects in milk (Fellman et al., 1991; Whited et al., 2002). Recently, Yeh et al. (2017a) demonstrated that vitamin concentrates used for fluid milk fortification had distinct carrot, fruity, citrus, rancid oil and painty aroma profiles indicating vitamin premixes contributed to off-flavors in milk.

Carotenoids, the precursor of vitamin A, are naturally present in milk and dispersed in the milk fat, which is simultaneously oxidized under light (Heyndricks et al., 2010). Carotenoids are also affected by the presence of riboflavin (Jung et al., 1998). Oxidation of carotenoids depends on oxygen availability and the carotenoid structure (Rodriguez-Amaya, 2016). Initially, part of the all-\(E\)-carotenoids, the usual configuration in nature, is isomerized to the \(Z\)-forms and both the \(E\)- and \(Z\)- isomers are oxidized (Rodriguez-Amaya, 2016). Subsequent fragmentations result in a series of compounds, similar to those produced in lipid oxidation (Rodriguez-Amaya, 2016). Cleavages at different sites of the polyene chain can also directly produce low mass volatile compounds, such as aldehydes, ketones, alcohols, and hydrocarbons (Rodriguez-Amaya, 2016). There are three types of volatile compounds from the oxidation of carotenoids: short acyclic compounds, which is directly from polyene chain; cleaved acyclic from lycopene and \(\beta\)-carotene; and secondary volatile compounds from the cleavage of polyene chain (Rodriguez-Amaya, 2016).

The oxidation of vitamin A has significance in flavor quality of vitamin A fortified milk (Yeh et al., 2017a). The major source of off-flavor compounds in vitamin A fortified milk are
degradation products from vitamin premixes used for fortification (Yeh et al., 2017a). Yeh et al. (2017a) determined eight of the key off-flavor compounds, including β-cyclocitral, β-ionone, β-damascenone, and α-irone, from vitamin premixes, which were characterized by carrot, fruity, rancid oil and painty, and four of those compounds were above sensory threshold in the fortified milks. Those compounds are cyclic derivatives formed from sequential cleavage of β-carotene. Depending on the cleavage sites of double bonds in the presence of oxygen, different types of volatile compounds can be formed: β-cyclocitral, β-ionone, β-damascenone, and/or secondary low mass volatile compounds. Carotenoid degradation is likely to increase with severe processing conditions, inadequate storage, permeability of packaging material to oxygen, and exposure to light (Rodriguez-Amaya, 2016).

Vitamin D is susceptible to degradation by oxygen, heat, and light (Yeh et al., 2017b). Renken and Warthesen (1993) found that vitamin D was lost in skim milk containing riboflavin after storage in the presence of oxygen and light. King and Min (1998) demonstrated that photosensitized singlet oxygen oxidation of vitamin D$_2$ occurred when both vitamin D$_2$ and riboflavin were exposed to light in the presence of oxygen. Light activates riboflavin, which generates singlet oxygen from triple oxygen and can react with the conjugated triene structure of vitamin D forming vitamin D-5,6-epoxide (King and Min, 2002).

**Effect of packaging materials.** There are two main factors related to packaging that affect flavor of milk: barrier to oxidation (from light and/or oxygen) and the migration from packaging material itself. The light transmission of various packaging materials can have significant effects on milk flavor due to the loss of riboflavin and lipid oxidation by light exposure during storage (Webster et al., 2009). Light-induced off-flavor was very common when milk was bottled in
clear bottles (Reineccius, 2006), and still remains a primary off-flavor for milks packaged in coextruded high density polyethylene (HDPE) jugs. The majority of U.S. fluid milk products are packaged in containers made of paperboard or HDPE (Whited et al., 2002). Packaging material can have a protective effect on milk quality through blocking or reducing the transmission of certain light wavelengths (Webster et al., 2009). However, the percentage of light transmission and oxygen permeation varies from traditional glass bottles to cartons and all modernized plastic or polyethylene materials (Dimick, 1982). Unlike paperboard which does offer light protection, common packaging materials, such as HDPE or clear polyethylene terephthalate (PET) cannot sufficiently protect milk from light under retail storage conditions (Johnson et al., 2015). Display cabinets for milk in retail outlets have a mean light intensity of 1000 lux, which is lower than daylight but still affects off-flavor development in milk within 24 hr (Konotominas, 2010). Clear glass transmits FL the most (91%), followed by clear polycarbonate (90%), PET (75-85%), HDPE (57-60%), and paperboard (4%) (Dimick, 1982; van Aardt et al., 2005). According to Cladman et al. (1998), milk products in HDPE had the largest vitamin A loss compared to PET, PET with a UV blocker, and low-density polyethylene (LDPE).

Wavelengths responsible for off-flavor formation in milk are smaller than 500 nm, and most transparent packaging materials offer inadequate protection against harmful wavelengths (400-500 nm) to milk (Konotominas, 2010). Colored packaging is capable to block wavelengths up to 500 nm (Konotominas, 2010). Black is the most effective in blocking wavelengths, used as a middle ‘buried’ layer in three-layer coextruded HDPE bottles for UP milk (Konotominas, 2010). Riboflavin destruction by light has been shown to be higher in skim milk than in whole milk, since harmful wavelengths can penetrate deeper into skim milk due to the absence of fat (Robertson, 1993; Cladman et al., 1998).
Flavor migration is the transfer/diffusion of soluble or volatile compounds of the packaging material to the contained products (Konotominas, 2010). Common migration substances are plastic additives (plasticizers, antioxidants, stabilizers, lubricants, etc.), solvents (adipic acid, toluene, ethyl acetate, etc.), monomers and oligomers (styrene, ethyl benzene, etc.), and environmental contaminants (naphthalene). Mainly low mass compounds are capable to migrate from these substances, often affecting the sensory properties (Konotominas, 2010). Those compounds include styrene monomers from polystyrene containers (Hauschild and Spingler, 1988), ethyl acetate from adhesives, and naphthalene from LDPE bottles (Lau et al., 1994). Several factors, such as fat content, time of contact, and storage temperature can affect the migration of these compounds (Konotominas, 2010). O'Neill et al. (1994) demonstrated that styrene migration was increased by higher fat content in milk. Naphthalene concentration in LDPE milk increased over storage time (Lau et al., 1994).

Non-thermal Processing of Milk

Recently, new preservation techniques have been considered as alternation for heat treatment in order to have a minimal effect on flavors and colors (Pereda et al., 2009; Chugh et al., 2014; Zhang et al., 2010). Non-thermal treatment not only result in microbial inactivation, but can also improve rennet or acid coagulation of milk without detrimental effects on important quality characteristics, such as flavor, vitamins, and nutrients (Trujillo et al., 2002; Kühn et al., 2006). The utilization of high pressure as a preservation method for milk was first reported by Hite (1899). Since then, a number of latest non-thermal processing methods, such as high-hydrostatic pressure (HHP), high pressure homogenization (HPH), pulsed electric field treatment (PEF), ultra-high pressure homogenization (UHPH), and microfiltration (MF) have been applied.
to milk and other liquid products to minimize the loss of quality and flavor change (Cadwallader and Singh, 2009; Chugh et al., 2014; Corbo et al., 2009), and to achieve shelf life (Trujillo et al., 2002; Zhang et al., 2010)). HHP is one of the most promising emerging methods due to its potential for shelf life extension with no severe compound degradation or MR (Trujillo et al., 2002). In the HHP system, foods are processed in a batch (solid products) or continuous system (liquid) in a pressure range of 50-1000 MPa. Temperature during pressure treatment can be from below 0 to above 100°C, while exposure time usually ranges from sec to 20 min (Corbo et al., 2009). Comparing the volatile profile of milk by HHP and conventional heat treatment, HPP at 25°C had a minimum change of volatile compounds in milk and HHP at 60°C formed aldehydes, whereas HTST generated both aldehydes and methyl ketones in milk (Vazquez-Landaaverde et al., 2006b). HPH induced a structural rearrangement of protein and an increased exposure of their hydrophobic regions (Vannini et al., 2004). The antimicrobial activity of HPH could be due to the disruption of the cell wall and outer membrane (Vannini et al., 2004).

PEF is based on short electric pulses of high voltage applied to a product that is placed between a pair of electrodes; microorganisms are inactivated by dielectric breakdown of the cell membrane without significant loss of food flavor and color (Corbo et al., 2009). UHPH is an emerging technology which combines high pressure with minimal temperature effect during processing. It is based on the same principle as conventional homogenization but works at higher pressures (up to 400 MPa) (Pereda et al., 2008). Although it is described as a promising technology to replace traditional heat treatments, milk temperature can be increased during UHPH treatment (19°C per 100 MPa) as a consequence of the adiabatic heating generated in the machine in addition to the high turbulence, shear and cavitation forces that the fluid suffers in the homogenization valve (Thiebaud et al., 2003). UHPH milk had less heat-related and oxidized
flavors compared to pasteurized milk and UHT milk (Pereda et al., 2008). However, more research is required to understand the effect of UHPH treatment on milk flavor.

Microfiltration (MF) is a membrane-driven separation process which has applied to remove bacteria and fat, and separate milk proteins (casein micelles and serum) (Elwell and Barbano, 2006). Although thermal processes can extend the shelf life of milk with respect to bacterial growth, most processors use extensive temperature and hold time that are above the minimum requirements in the PMO (Douglas et al., 2000). MF in combination with HTST has been applied as an alternative to UP for extending refrigerated milk shelf life without distinct cooked flavor of UP milk. (Elwell and Barbano, 2006). In this technology, MF of whole milk is not feasible as the particle size distribution of bacterial cells and spores is similar to that of milk fat globules (Rysstad and Kolstad, 2006). Thus, the milk needs to be centrifugally separated, and only the skim milk can go through MF. On the other hand, different ceramic membranes with pore diameters can be used to minimize changes in milk composition as the particle size distribution of cells and spores can be overlapped with casein micelles (Rysstad and Kolstad, 2006). Pore diameters of 0.8-1.4 µm are commonly used, and this size allows bacteria to pass through the membrane (Rysstad and Kolstad, 2006). Pore size of about 1.4 µm can achieve the right balance between the rejection of the bacteria and the long-term flux with little or no rejection of other milk components (Pafylias et al., 1996). MF with a 1.4 µm membrane has provided complete removal of the SCC from skim milk (Saboya and Maubois, 2000; te Giffel and van der Horst, 2004). After the bacteria are concentrated in the MF retentate, the final milk (MF skim permeate) is nearly bacteria-free but still must receive a minimum HTST treatment to ensure the elimination of vegetative pathogens (Elwell and Barbano, 2006; Rysstad and Kolstad,
The major advantages of MF milk are a longer shelf life by bacterial removal and the maintained flavor quality of fresh milk due to minimal heat treatment (Pafylis et al., 1996).

The comparison of volatile profiles between thermal and non-thermal processing has been observed by only a few studies (Pereda et al., 2008; Zhang et al., 2010; Chugh et al., 2014). Heat treated milk had increased aldehydes and methyl ketones, while UHPH and PEF milk had increased aldehydes only (Pereda et al., 2008; Zhang et al., 2010). Although the concept of MF milk was introduced in the late 1980s (Piot et al., 1987; Holm et al., 1986), there is a lack of studies on flavor aspects of MF milk. Chugh et al. (2014) reported MF and PEF are potential technologies to produce good sensory quality milk followed by no significant changes of volatiles compared to HTST. However, the effect of non-thermal processing of milk on sensory properties or flavor chemistry has not yet been fully understood.

Flavor Analysis of Milk

The classical approach in flavor analysis starts from isolating volatiles from milk and includes different extraction methods: solvent (e.g., solvent extraction, vacuum distillation, and SAFE) and solvent-less way (e.g., SPME and SBSE). Some modern microextraction techniques, such as SPME and SBSE, now allow the capture of these compounds by direct or headspace adsorption from food, avoiding time-consuming steps and have appealing advantages in terms of shortened analysis time, easier handling, and higher sensitivity (Vazquez-Landaverde et al., 2006; Jeleń et al., 2012). SPME is an extraction technique that uses a fused silica fiber that is coated on the outside with a different stationary phase (Kataoka et al., 2000). It is the most predominant method for characterizing volatiles or flavors (Jeleń et al., 2012), and has been used routinely in combination with gas chromatography (GC) and gas chromatography-mass
spectrometry (GC–MS). SBSE is a relatively new method, which uses a stir bar coated with polydimethylsiloxane (PDMS), and is capable to absorb 50-250 times the volatiles of SPME exposed in the headspace or liquid sample (Jeleń et al., 2012). Since it carries a high volume of phase in stir bar resulting in lower detection limits, it can be an ideal tool for off-flavor analysis (Jeleń et al., 2012).

Flavor analysis in milk has been conducted extensively for many years since milk and milk products are recognized as an important part of daily nutrition (Cadwallader and Singh, 2009). Due to the low concentrations of flavors in milk and the complexity of the milk emulsion (Friedrich and Acree, 1998; Havemose et al., 2007), isolating flavor compounds from milk is challenging and several methods have been applied to extract flavor compounds from different types of milk. Previous studies extracted volatiles from milk using dynamic headspace (Marsili and Miller, 1998), purge and trap (Valero et al., 2001; Contraini and Povolo, 2002), cold trap (Contarini et al., 1997), steam vacuum distillation (Jeon et al., 1978), solvent-assisted flavor evaporation (SAFE, Colahan-Sederstrom and Peterson, 2005; Kokkinidou and Peterson, 2014; Havemose et al., 2007), and solid-phase microextraction (SPME, Pereda et al., 2008; Vazquez-Landaverde et al., 2005; Marsili, 1999; Karatapanis et al., 2006; Mestdagh et al., 2005; Havemose et al., 2007; Mounchili et al., 2005; Contraini and Povolo, 2002; Marsili, 2000).

GC-MS has frequently been used for analyzing the volatile compounds of many dairy products because it can collect mass spectra to identify compounds. GC separation is coupled with capillary columns and a MS detector, is able to obtain the mass spectral information (e.g., exact mass of molecular ion, or fragmentation patterns) as mass to charge scale (m/z), and is the most universal tool for flavor analysis, depending on the volatility and the polarity of the analytes (Mariaca and Bosset, 1997). Two MS methods, full-scan and selected ion monitoring
(SIM) mode, are commonly applied to identify the chemical structure of unknown compounds (Mariaca and Bosset 1997). With scan mode, the mass spectra are matched with the standards or compared to public and commercial databases of reference spectra, and then identified. In SIM mode, however, only target ions of compounds of interest are screened and quantified with high selectivity and sensitivity. The primary advantages of GC are its high separation efficiency and reproducible retention times, which can be compared with the retention index (RI), and those of MS are sensitivity and standardized mass spectral fingerprints. Ideally, GC-MS based technique allows the simultaneous analysis of several hundred compounds and covers a wide range of compounds, even non-volatiles, through the derivatization process. However, GC-MS has limitations on estimating important flavor compounds in foods. In other words, it does not guarantee that all the volatile compounds identified by GC-MS are odor-active compounds (Grosch, 1993; Friedrich and Acree, 1998). According to Grosch (2001), it is estimated that less than 5% of volatile compounds play a significant role in the formation of aroma in foods. Therefore, flavor analysis should be focused on flavor compounds, which must be distinguished from volatile compounds that do not have a role in flavors (Grosch, 1993; Friedrich and Acree, 1998). As such, sensory analysis must play a role in any chemical analysis of flavor.

Sensory-directed approaches, such as olfactometric analysis and sensory analysis along with advanced instrumental technique have been applied to evaluate key aroma active compounds in milk and dairy products (Cadwallader and Singh, 2009; Drake et al., 2007a; Boelrik and De Jong 2003). GC-Olfactometry (GC-O) is the technique that combines olfactometry and the human nose as a detector with GC. It is based on the concept that compounds present above orthonasal sensory threshold concentrations in foods are more likely to be important compounds to aroma and flavor (Grosch, 1993). To quantify and determine odor
perception at the sniffing port by each panelist, a few different methods are generally recognized, i.e. combined hedonic aroma response measurement (CHARM) developed by Acree et al. (1984), and aroma extract dilution analysis (AEDA), introduced by Ullrich and Grosch (1987). The basis is quite similar in that sniffing serial dilutions of an extract through the GC run is done, but the values are represented differently. In the case of AEDA, the highest dilution indicates the value where an odor-active component may still be detected based on the flavor dilution (FD) factor (Grosch, 1993). Despite the major challenge that odor-detecting capacities or thresholds vary greatly among individuals (Friedrich and Acree, 1998), the GC-O sniffing technique allows the confirmation of important flavors (Grosch, 2001). For this reason, several studies have applied GC-O to characterize flavors in milk (Moio et al., 1993; Moio et al., 1994) and off-flavors in milk (Mouchili et al., 2005). In particular, Moio et al. (1994) demonstrated nine odor active compounds from pasteurized milk including heptanal, indole, nonanal, 1-octen-3-ol, dimethyl sulfone, hexanal, 2-nonanone, benzothiazole, and δ-decalactone.

Sensory analysis can be much more complicated to apply in flavor research, compared to instrumental analysis. As far as flavor is concerned, both smell and taste should be considered. In sensory-directed flavor analysis, sensory techniques can be used to establish relationships between sensory and instrumental measurements, and enhance the understanding of the consumer (Drake, 2007b). Also, it plays a critical role in interpreting and identifying compounds responsible for specific odors (key aroma compounds, key odorants) in foods (Jeleń et al., 2012). Target analysis is aimed to quantify the targeted group of flavor compounds of interest (e.g., fatty acids, sulfur-containing compounds, etc.) as well as compounds causing quality problems (Jeleń et al., 2012) and to understand mechanisms of action or markers. For these reasons, targeted approaches have been used to characterize flavors from the effects of heat.
treatment (Contarini et al., 1997), packaging materials (Smet et al., 2009; Mestdagh et al., 2005), storage condition, antioxidants (Van Aardt et al., 2005a; Van Aardt et al., 2005b; Vazquez-Landaverde and Qian, 2007), and aroma inhibition (Colahan-Senderstrom and Peterson, 2005; Troise et al., 2014; Kokkinidou and Peterson, 2014) in different types of milk. Often, fundamental multivariate statistics, such as principal component analysis (PCA) or partial least squares (PLS) can be combined to visualize and distinguish compounds by given factors (e.g., processing, nutrient compositions, etc.). Consequently, off-flavor studies can be a combination of targeted analysis with statistic treatment, by predicting quality, clustering patterns of off-flavors, and estimating desirable flavors in milk (Marsili and Miller, 1998; Marsili, 1999; Marsili and Miller, 2002).

A recent trend in flavor chemistry studies of foods is the combination of multiple methods for extended coverage of different classes of compounds (Vrhovesk et al., 2014). Ideally, this approach leads to all classes of different compounds and can be included in a single run using GC coupled with different detectors, with various sample extraction techniques (Vrhovesk et al., 2014). In flavor analysis, the use of microextraction techniques combined with GC triple quadrupole MS (GC-MS/MS) have been introduced with wine (Langen et al., 2013; Slabizki et al., 2016), fruits (Vrhovesk et al., 2014), rice (Hopfer et al., 2016), tobacco (Zhang et al., 2013), and Perilla seed oil (Kwon et al., 2013). The increase in sensitivity of MS/MS can enhance separation power of targeted analysis (Hjelmeland et al., 2012; Langen et al., 2013), and be applied to the trace quantification of a number of flavor compounds in food products. A strategy of using several sequential approaches based on SPME and SBSE in conjunction with GC-MS/MS must be an effective tool to develop a comprehensive flavor chemistry of milk.
Once key aroma active compounds have identified, a model mixture with all the potential precursors occurring in the food can be reacted under simulated conditions to determine and clarify the reaction pathways leading to those compounds (Shieberle, 2005; Cerny, 2008). Model reactions with $^{13}$C-labeled precursors are useful for obtaining information about the origin of the carbons in the flavor compounds (Cerny, 2008). In traditional approaches, an appropriately labeled precursor was reacted under Maillard type conditions, and mostly carbohydrates labeled at carbon-1 were used (Shieberle, 2005). An increase in the molecular mass by one unit served as proof that the label had been introduced in the target molecule (Shieberle, 2005). This concept, implying the validity of a single formation pathway, has been used to clarify Maillard type reactions of, for example, isoleucine (Tressl et al., 1995), proline (Tressl et al., 1993), or alanine (Yaylayan and Keyhani, 2000). More recently, using a mixture of unlabeled and fully labeled carbohydrates has been introduced by Shieberle (2005), which generates a mixture of isotopomers of the target molecule that can be deduced based on mass spectroscopic data (Shieberle, 2005; Cerny, 2008).

The flavor of milk is imparted by the natural components, and its profile and composition are attributed to various factors that were reviewed previously. Key changes in milk flavor are generated during thermal processing of milk the most, associated with the Maillard reactions (Van Boekel, 1998; Colahan-Sederstrom and Peterson, 2005; Kokkinidou and Peterson, 2014; Troise et al., 2014), lipid degradation (Calvo and Hoz, 1992), and thermal denaturation of serum (whey) protein and other proteins in the MFGM (Mehta, 1980). Those compounds include various groups of compounds: ketones, alcohols, esters, volatile free fatty acids, sulfur compounds, and other miscellaneous compounds (Table 1.5). As seen in Table 1.5, most compounds were present in nearly all milks, suggesting that the difference in flavor profile is
more likely originated from concentration differences of a set of these compounds rather than the absence/presence of specific compounds (Cadwallader and Singh, 2009). The difference is more likely derived from excessive heat treatment, such as UP and UHT, where distinct cooked and sulfur compounds are generated (Colahan-Senderstrom and Peterson, 2005; Vazquez-Landaaverde et al., 2006; Jo et al., 2018). Most previous studies investigated flavor of UHT milk because of its characteristic distinct flavor compared to HTST milks.

Despite the considerable progress made in milk flavor studies, flavor comparisons between different continuous heating methods of UP or UHT verses HTST have not yet been fully understood. Recently, Jo et al. (2018) investigated the impact of different heat treatments on flavor differences between HTST versus UP with DSI and IND. The study demonstrated that the impact of IND and DSI on flavor profile of milk were distinguished; IND was more related to compounds derived from sugar degradation, MR, and protein denaturation, which explains the higher sweet aromatic and cooked flavors in IND-UP milk, whereas DSI was highly associated with sulfurous compounds, which likely impact sulfur/eggy and cooked flavors in DSI-UP milk. It would be expected that both IND and DSI are distinguished from HTST milks having higher cooked and sulfur flavors due to excessive heat. However, it is also important to note that flavor profiles of IND and DSI were different because these methods have significant differences in temperature-time profiles (Lee et al., 2017). The Maillard reaction and protein denaturation during thermal processing affected the flavor quality of UP milks and distinct sensory and chemical differences between DSI-UP and IND-UP milks. Future work should continue to investigate the reaction pathways of sulfur amino acids present in milk protein as a precursor of sulfur compounds and their role in UP-IND and UP-DSI milks.
Conclusion

Flavors in milk are generally thought to be fresh and bland. However, noticeable off-flavors often cause consumer dissatisfaction. Milk manufacturing involves both spontaneous and process induced reactions, and breakdown products can produce off-odors, new flavors, loss of nutrient content, and color deterioration. Consequently, flavor is the primary matter of concern regarding the quality of milk. It is obvious that current flavor analysis has become more precise and rapid along with all the advantages of different techniques and approaches, still yet, the majority of flavor studies so far have been focused on aroma/odor compounds. A key aspect in flavor research of milk is elucidating mechanisms of action or markers by interpreting relevant studies and data. By building the data for multivariate analysis as part of flavor research, it is possible to establish links between initiative reaction markers and related circumstances. A strategy of using several sequential approaches (e.g., the GC-MS, sensory-directed analysis, and multivariate statistical analysis) will be needed to maximize coverage of flavors that are present in milk.
REFERENCES


Table 1.1. Major fatty acids found in milk triglycerides and their related flavor compounds (modified from MacGibbon and Taylor, 2006; Fox, 2009; Jensen, 2002)

<table>
<thead>
<tr>
<th>Origin</th>
<th>Name</th>
<th>Carbon number</th>
<th>Average range (wt%)</th>
<th>Involved flavor compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing β-hydroxy butanoic acid, which is synthesized by bacteria in the rumen</td>
<td>Butanoic</td>
<td>C4:0</td>
<td>2-5</td>
<td>Butanoic acid</td>
</tr>
<tr>
<td>Synthesized in the mammary gland from acetyl CoA</td>
<td>Caproic</td>
<td>C6:0</td>
<td>1-5</td>
<td>Caproic acid</td>
</tr>
<tr>
<td></td>
<td>Caprylic</td>
<td>C8:0</td>
<td>1-3</td>
<td>Caprylic acid, 2-heptanone</td>
</tr>
<tr>
<td></td>
<td>Capric</td>
<td>C10:0</td>
<td>2-4</td>
<td>Capric acid, 2-nonanone</td>
</tr>
<tr>
<td></td>
<td>Lauric</td>
<td>C12:0</td>
<td>2-5</td>
<td>Lauric acid</td>
</tr>
<tr>
<td></td>
<td>Myristic</td>
<td>C14:0</td>
<td>8-14</td>
<td></td>
</tr>
<tr>
<td>50% are from acetyl CoA and 50% are from diet</td>
<td>Palmitic</td>
<td>C16:0</td>
<td>22-35</td>
<td>Heptanal</td>
</tr>
<tr>
<td>Obtained from diet</td>
<td>Palmitoleic</td>
<td>C16:1</td>
<td>1-3</td>
<td></td>
</tr>
<tr>
<td>Produced from C18:0 in the liver Δ-9 desaturase</td>
<td>Stearic</td>
<td>C18:0</td>
<td>9-14</td>
<td>Octanal, nonanal, decanal, 2-decenal, 2-undecenal, γ-decalactone, δ-dodecalactone, γ-dodecanolactone</td>
</tr>
<tr>
<td></td>
<td>Oleic</td>
<td>C18:1</td>
<td>20-30</td>
<td></td>
</tr>
<tr>
<td>10% are from diet or produced from other unsaturated fatty acids</td>
<td>Linoleic</td>
<td>C18: 2</td>
<td>1-3</td>
<td>Hexanal, 2-heptenal, 2-octenal, 3-nonenal, 2-nonenal, 2,4-decadienal, 2-pentylfuran, δ-decalactone, γ-decalactone, γ-nonalactone, (Z)-6-γ-dodecenelactone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C18: 3</td>
<td>0.5-2</td>
<td>Propanal, 3-hexenal, 2-hexenal, 2-pentenal, 2,4-heptadienal, 2,5-octadienal, 3,6-nonadienal, 2,4,7-decatrienal, 1,5-dien-3-one, 3-hexenal, 4-heptenal</td>
</tr>
</tbody>
</table>
Table 1.2. Major enzymes in milk (modified from Walstra et al., 1999; Campbell and Drake, 2013)

<table>
<thead>
<tr>
<th>Origin</th>
<th>Enzymes</th>
<th>Optimum pH</th>
<th>Optimum Temperature</th>
<th>Inactivation</th>
<th>Effects on milk quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk Fat globule membrane</td>
<td>Xanthine oxidase (EC 1.17.3.2)</td>
<td>8</td>
<td>37°C</td>
<td>7 min at 73°C</td>
<td>Serves as a source of superoxide anion in bovine milk and creates flavor deterioration</td>
</tr>
<tr>
<td></td>
<td>Alkaline phosphatase (EC 3.1.3.1)</td>
<td>9</td>
<td>37°C</td>
<td>20 sec at 73°C</td>
<td>Be used as an indicator of proper milk pasteurization</td>
</tr>
<tr>
<td>Casein micelle</td>
<td>Lipoprotein lipase (EC 3.1.1.34)</td>
<td>9</td>
<td>37°C</td>
<td>30 sec at 73°C</td>
<td>Liberates free fatty acids by enzymatic hydrolysis of triglyceride</td>
</tr>
<tr>
<td></td>
<td>Plasmin (EC 3.4.21.7)</td>
<td>8</td>
<td>37°C</td>
<td>40 min at 73°C</td>
<td>Breaks down casein (β-casein) in milk</td>
</tr>
<tr>
<td>Serum</td>
<td>Lactoperoxidase (EC 1.11.1.7)</td>
<td>6.5</td>
<td>20°C</td>
<td>10 min at 73°C</td>
<td>Catalyzes the oxidation of molecules in the presence of hydrogen peroxide, and has antimicrobial activity</td>
</tr>
<tr>
<td>Plasma</td>
<td>Sulphydryl oxidase (EC 1.8.3-)</td>
<td>7</td>
<td>35°C</td>
<td>3 min at 73°C</td>
<td>Catalyzes the oxidation of sulphydryl groups</td>
</tr>
<tr>
<td></td>
<td>Superoxide dismutase (EC 1.15.1.1)</td>
<td>7.8</td>
<td>25°C</td>
<td>70 min at 76°C</td>
<td>Catalyzes the dismutation of superoxide anion, and could play an important role in preventing lipid oxidation of milk</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>Catalase (EC 1.11.1.6)</td>
<td>7</td>
<td>37°C</td>
<td>16 sec at 65°C</td>
<td>Decomposes hydrogen peroxide</td>
</tr>
</tbody>
</table>
Table 1.3. Comparison of indirect heating and direct heating for ultrapasteurization (UP) and ultra-high treatment (UHT)

<table>
<thead>
<tr>
<th>Heating type</th>
<th>Indirect heating</th>
<th>Direct heating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feature</td>
<td>Heating medium and product are not in direct contact</td>
<td>Steam has intimate contact with product</td>
</tr>
<tr>
<td></td>
<td>Two media are kept separate</td>
<td>Use culinary steam to heat a product quickly</td>
</tr>
<tr>
<td></td>
<td>Heat transfer is different from the type of medium</td>
<td>Requires a higher pressure to maximize the heat transfer</td>
</tr>
<tr>
<td></td>
<td>Takes up more space</td>
<td>Less floor space but requires a chamber</td>
</tr>
<tr>
<td></td>
<td>Longer heating and cooling</td>
<td>Short holding time and gentle heating</td>
</tr>
<tr>
<td>Impact on milk</td>
<td>Possible burn-on or browning because of the large surface area</td>
<td>Lack of overheating or burn-on</td>
</tr>
<tr>
<td>Medium</td>
<td>Plate exchanger</td>
<td>Added moisture must be removed by vacuum flash chamber</td>
</tr>
<tr>
<td></td>
<td>Tubular exchanger</td>
<td>Steam infusion</td>
</tr>
<tr>
<td></td>
<td>Scraped surface exchanger</td>
<td>Steam injection</td>
</tr>
</tbody>
</table>
Table 1.4. Comparison of analysis of 5-hydroxymethylfurfural (HMF) and furosine as a marker of thermal damage in milk products

<table>
<thead>
<tr>
<th></th>
<th>5-hydroxymethylfurfural (HMF)</th>
<th>Furosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis technique</td>
<td>Spectrophotometric (the most common)</td>
<td>HPLC (the most preferred)</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>CZE</td>
</tr>
<tr>
<td>Key analytical step</td>
<td>Preliminary extraction normally performed with solid phase</td>
<td>Preliminary acid hydrolysis for 20h to have</td>
</tr>
<tr>
<td></td>
<td>extraction</td>
<td>high recovery of furosine (avoiding furosine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>conversion to N-ε-(carboxymethyl)lysine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acid hydrolysis of the Amadori compound to</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lysine (40%), furosine (32%) and a small</td>
</tr>
<tr>
<td></td>
<td></td>
<td>amount of pyridosine</td>
</tr>
<tr>
<td>Preferred wavelengths</td>
<td>Ultraviolet detection at 280, 284 or 285 nm</td>
<td>Ultraviolet detection at 280 nm</td>
</tr>
<tr>
<td>Advantages</td>
<td>A classic heating or process-control parameter</td>
<td>A direct marker of lysine reaction products</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A representative marker of Amadori products</td>
</tr>
<tr>
<td></td>
<td></td>
<td>from the early stage of MR that are</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nutritionally unavailable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reproducible recovery rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blocked lysine can be calculated</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>Not only formed from MR but also from the isomerization and</td>
<td>Formed from Amadori products with a</td>
</tr>
<tr>
<td></td>
<td>subsequent degradation of sugars</td>
<td>yield of 30-40%</td>
</tr>
<tr>
<td></td>
<td>Low accuracy and sensitivity by possible interference by non-</td>
<td>Uncertainty of the conversion factor of the</td>
</tr>
<tr>
<td></td>
<td>HMF analytes having same wavelength area in spectrophotometric</td>
<td>Amadori product to furosine</td>
</tr>
<tr>
<td></td>
<td>system</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low detection limit by HPLC system</td>
<td>Furosine decreases in severely heated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>products after long storage periods</td>
</tr>
</tbody>
</table>
Table 1.5. Aroma active compounds of milk positively identified by gas chromatography-mass spectrometry (GC-MS)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Milk type</th>
<th>Raw</th>
<th>HTST</th>
<th>UP</th>
<th>UHT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sulfur compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogen sulfide</td>
<td></td>
<td>1</td>
<td>1, 20</td>
<td>17, 20</td>
<td>1, 14, 16</td>
</tr>
<tr>
<td>Methanethiol</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1, 14</td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
<td></td>
<td>4, 19</td>
<td>4, 14, 20</td>
<td>17, 20</td>
<td>1, 3, 4, 13, 14, 16</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td></td>
<td>1</td>
<td>1, 14, 20</td>
<td>17, 20</td>
<td>1, 14, 16</td>
</tr>
<tr>
<td>Dimethyl disulfide</td>
<td></td>
<td>1</td>
<td>1, 14, 18, 20</td>
<td>17, 20</td>
<td>1, 3, 4, 13, 14, 16</td>
</tr>
<tr>
<td>Dimethyl trisulfide</td>
<td></td>
<td>1</td>
<td>1, 20</td>
<td>17, 20</td>
<td>1, 16</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td></td>
<td>1</td>
<td>1, 14</td>
<td>20</td>
<td>1, 14, 16</td>
</tr>
<tr>
<td>Dimethyl sulfone</td>
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Table 1.5. Continued

a The compounds were previously identified in milk by the authors given in parenthesis; 1 (Vazquez-Landaverde et al., 2006); 2 (Belitz et al., 2009); 3 (Valero et al., 2001); 4 (Vazquez-Landaverde et al., 2005); 5 (Potineni and Peterson, 2005); 6 (Moio et al., 1994); 7 (Czerny and Schieberle, 2007); 8 (Marsili, 1999); 9 (Kokkinidou and Peterson, 2014); 10 (Jeon et al., 1978); 11 (Colahan-Senderstrom and Peterson, 2005); 12 (Troise et al., 2014); 13 (Contarini et al., 1997); 14 (Mestdagh et al., 2005); 15 (Scanlan et al., 1968); 16 (Al-Attabi et al., 2014); 17 (Simon et al., 2001); 18 (Hougaard et al., 2011); 19 (Mounchili et al., 2005); 20 (Jo et al., 2018).

b Literature numbers 1-8 = UHT milk purchased from retail (heating type not addressed); 9-12 = UHT milk processed in lab or pilot scale (heating type not addressed); 13 = UHT milk processed by direct steam injection; 14-16 = UHT milk processed by indirect heating; 17 = UP milk processed by indirect heating.
Figure 1.1. Structure of triglycerides- \( sn \)- positions.
Figure 1.2. Fractions of major milk proteins.
Figure 1.3. Structure of lactose.
Figure 1.4. Structure of lactulose.
Figure 1.5. Schematic overview of the early Maillard reaction in milk leading to the Amadori product (taken from van Boekel, 1998).
Figure 1.6. Degradation of the Amadori product (taken from van Boekel, 1998).
Figure 1.7. Breakdown of the Amadori product in the advanced Maillard reaction under acidic, neutral and basic conditions (taken from van Boekel, 1998).
Figure 1.8. Formation of dimethyl sulfide via Strecker degradation of methionine and the oxidation of methanthiol (taken from Zabbia et al., 2012).
Figure 1.9. Formation of aldehydes via lipid oxidation (taken from Zabbia et al., 2012).
Figure 1.10. The chemical mechanism for the formation of singlet oxygen in the presence of sensitiser, light and triplet oxygen showing the excitation and deactivation of photosensitizer in the formation of singlet oxygen (taken from Min and Boff, 2002).
Figure 1.11. Proposed riboflavin catalyzed Strecker degradation-like reaction of methionine to form methional (taken from Min and Boff, 2002).
CHAPTER 2: FLAVOR AND FLAVOR CHEMISTRY DIFFERENCES AMONG MILKS PROCESSED BY HIGH TEMPERATURE SHORT TIME OR ULTRAPASTEURIZATION

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The content of this chapter was published in:

Abstract

Typical high temperature short time (HTST) pasteurization encompasses a lower heat treatment and shorter refrigerated shelf life compared with ultrapasteurization (UP) achieved by direct steam injection (DSI-UP) or indirect heat (IND-UP). A greater understanding of the effect of different heat treatments on flavor and flavor chemistry of milk is required to characterize, understand, and identify the sources of flavors. The objective of this study was to determine the differences in the flavor and volatile compound profiles of milk subjected to HTST, DSI-UP, or IND-UP using sensory and instrumental techniques. Raw skim and raw standardized 2% fat milks (50 L each) were processed in triplicate and pasteurized at 78°C for 15 s (HTST) or 140°C for 2.3 s by DSI-UP or IND-UP. Milks were cooled and stored at 4°C, then analyzed at d 0, 3, 7, and 14. Sensory attributes were determined using a trained panel, and aroma active compounds were evaluated by solid-phase micro-extraction or stir bar sorptive extraction followed by gas chromatographymass spectrometry, gas chromatography-olfactometry, and gas chromatography-triple quad mass spectrometry. The UP milks had distinct cooked and sulfur flavors compared with HTST milks. The HTST milks had less diversity in aroma active compounds compared with UP milks. Flavor intensity of all milks decreased by d 14 of storage. Aroma active compound profiles were affected by heat treatment and storage time in both skim and 2% milk. High-impact aroma active compounds were hydrogen sulfide, dimethyl trisulfide, and methional in DSI-UP and 2 and 3-methylbutanal, furfural, 2-heptanone, 2-acetyl-1-pyrroline, 2-aminoacetophenone, benzaldehyde, and dimethyl sulfide in IND-UP. These results provide a foundation knowledge of the effect of heat treatments on flavor development and differences in sensory quality of UP milks.

Key words: fluid milk, ultrapasteurization, flavor
Introduction

The flavor of dairy products is a critical parameter affecting consumer acceptance, shelf life, and other attributes (Kühn et al., 2006; Drake et al., 2007). For fluid milk, flavor is initially influenced by raw milk quality, such as microbial counts and heat-resistant enzymes present in milk (Barbano et al., 2006). When thermal treatment is employed to reduce or destroy the microbial load and enzyme activity to ensure safety and to increase shelf life, the flavor of the milk changes and differs from that of raw milk (Walstra et al., 1999). Typical milk processing in the United States involves HTST pasteurization (minimum of 72°C for 15 s), providing approximately 3 wk of shelf life (Boor, 2001). Ultrapasteurization (UP) and UHT are also thermal treatments for extended shelf life of milk (Boor, 2001; Colahan-Sederstrom and Peterson, 2005; Lee et al., 2017). The thermal processing conditions for UP milk are defined as at or above 138°C (280°F) for at least 2 s (21 CFR 131.3, FDA, 2017), and this product generally has an extended shelf life under refrigerated conditions. The UHT milk has the same thermal process as for UP milk, but the milk is aseptically packaged to be shelf stable for at least 6 mo without refrigeration (Boor, 2001). Processing by UP can be operated with 2 continuous heating methods: (1) direct heating, which quickly heats the milk by direct steam injection (DSIUP), followed by vacuum cooling to remove water from the steam, and (2) indirect heating (IND-UP), which heats the milk by a tubular, scraped surface heat exchanger or plate-heat exchanger (Datta et al., 2002; Lee et al., 2017). The UP milk receives a significantly higher thermal load than HTST milk shown by thermal damage indicators, such as furosine and serum protein denaturation (Lee et al., 2017). Lee et al. (2017) reported that IND-UP milks had the highest furosine content, followed by DSI-UP, and HTST. In contrast, UP milks had more serum protein denaturation than HTST, but IND-UP was more severe than DSI-UP (Lyster et al., 1971; Lee et
al., 2017). Due to differences in heat time, temperature, and total heat load, the sensory profiles of DSI-UP, IND-UP, and HTST milks were all distinct (Lee et al., 2017). The flavor of pasteurized milk is derived from various reactions during thermal processing or storage, which also affect color (Schamberger and Labuza, 2007). Key changes in flavor during thermal processing of milk are associated with Maillard reactions (Van Boekel, 1998; Colahan-Sederstrom and Peterson, 2005; Kokkinidou and Peterson, 2014; Troise et al., 2014), lipid degradation (Calvo and Hoz, 1992), and thermal denaturation of serum (whey) protein and other proteins in the milk fat globule membrane (Mehta, 1980). Lactose and amino groups in milk proteins undergo Maillard reactions during heat treatment, and this process generates a wide range of flavor compounds, such as Strecker aldehydes, sulfur- and nitrogen-containing compounds, maltol, and diacetyl (Calvo and Hoz, 1992; Van Boekel, 1998; Reineccius, 2006). Lipid degradation during thermal processing of milk induces β-oxidation of free fatty acids generating methyl ketones (Calvo and Hoz, 1992). β-Lactoglobulin in serum protein liberates volatile sulfur compounds by heat denaturation, which involves unfolding and exposure of reactive sulfides (Calvo and Hoz, 1992; Kühn et al., 2006). Volatile compounds are the source of the aroma portion of food flavor, but only a small fraction of volatile compounds present in food are flavor active and contribute to flavor (Grosch, 2001). In classical flavor analysis, studies were focused on a single component of flavor and dependent on isolation of volatile compounds by different extraction methods using solvents (e.g., solvent extraction, and solvent-assisted flavor evaporation), or headspace extraction [e.g., dynamic headspace, solid phase microextraction (SPME), and stir-bar sorptive extraction (SBSE; Reineccius, 2006; Jeleń et al., 2012; Park and Drake, 2016). These methods require time and several steps that often lead to the loss of volatile compounds (Vazquez-Landaverde et al., 2006). As flavor is a sensory perception,
volatile compounds confirmed to be aroma active by olfactometry methods are more likely to be important compounds to flavor (Reineccius, 2006). More recently, new approaches include the combination of multiple techniques or detectors to obtain different classes of compounds in a single run (Vrhovsek et al., 2014). The SPME combined with GC-triple quadrupole mass spectrometry (GC-MS/MS) has been introduced to flavor studies of wine (Mattivi et al., 2011; Hjelmeland et al., 2012; Langen et al., 2013; Slabizki et al., 2016), single rice kernel (Hopfer et al., 2016), Perilla seed oils (Kwon et al., 2013), fruits (Vrhovsek et al., 2014), and tobacco (Zhang et al., 2013) but has yet to be used in analyzing flavor compounds from milk. The flavor of UP milk is distinct from HTST and has been characterized as having distinct sulfurous, cooked, cabbage, and caramelized notes (Colahan-Sederstrom and Peterson, 2005; Vazquez-Landaverde et al., 2006; Lee et al., 2017). These distinct flavors are a drawback of UP milk and are a barrier to US consumer acceptance (Perkins and Deeth, 2001; Lee et al., 2017). Lee at al. (2017) recently demonstrated that both adults and children preferred the flavor of HTST milk over DSI-UP or IND-UP milks. Previous studies on the flavor chemistry of UP milk (Colahan-Sederstrom and Peterson, 2005; Potineni and Peterson, 2005; Kokkinidou and Peterson, 2014) have been conducted with indirect heat treatment with limited information on actual UP parameters or without comparison to traditional HTST milks. More importantly, no previous study to our knowledge has directly compared the flavor chemistry of HTST versus UP with DSI and IND in relation to understanding the nature of the differences between direct and indirect heat treatment when starting from the same batch of raw milk. The objective of our study was to develop a comprehensive flavor chemistry profile of milk processed by HTST, DSI-UP, and IND-UP and to identify compounds responsible for the flavor differences of these milks (key aroma compounds, and odorants). A collaborative strategy of pilot plant processing and several
sequential approaches for volatile compound extraction based on SPME and SBSE, in conjunction with GC-olfactometry (GC-O) followed by GC-MS, flame photometric detection (FPD), or MS/MS in conjunction with sensory analysis provided effective tools to characterize and compare the flavor profiles of milk.

**Materials and Methods**

*Milk Preparation*

Two fat levels (skim and 2%) and 3 different heat treatments (HTST, DSI-UP, and IND-UP) of milk were evaluated. Raw skim milk (200 L at 3.1% protein, 0.07% fat) and raw cream (45.4% fat) were obtained from the North Carolina State University dairy facility. The cream was separated from raw whole milk by a cold bowl separator (model 590, Separators Inc., Indianapolis, IN). Raw skim milk (100 L) was standardized to 2% fat milk with the raw cream. A microthermics EHVH pasteurization unit (Microthermics, Raleigh, NC) with a Microthermics Steam Injection Module, and a 2-stage homogenizer (model NS2006H, GEA Niro Soavi, Parma, Italy) was used for milk processing. Raw skim and raw 2% milks were processed by HTST, DSIUP, or IND-UP as described by Lee et al. (2017). For HTST treatment, raw milks were preheated at 60°C, homogenized and pasteurized at 78°C for 15 s at a flow rate of 2.0 L/min before cooling to 10°C. The DSI-UP milks were processed at a flow rate 1.2 L/min, preheated at 90°C, and heated to 140°C for 2.3 s by direct culinary steam injection (model LG-30, Electro-Steam Generator Corp., Alexandria, VA) using a Microthermics Steam Injection Module. Then milks were cooled to 85°C by vacuum cooling to remove heat and water added as steam. The DSI-UP milks were then homogenized and cooled to 10°C. For IND-UP, milks were preheated at 90°C and pasteurized at 140°C for 2.3 s at a 1.3 L/min flow rate. Milks were cooled to 85°C
before homogenization and then cooled to 10°C. All milks were packaged in light-shielded, half-gallon high-density polyethylene containers (Upstate Niagara Cooperative Inc., Buffalo, NY), stored at 4°C, and sampled at d 0, 3, 7, and 14 for analyses. The experiment was replicated in triplicate.

**Chemical Standards**

Chemical standards (no. 1–12, 14–29, and internal standards 1 and 2; Table 2.1) were purchased from Sigma-Aldrich (St. Louis, MO). A standard (no. 13; Table 2.1) was obtained from BOC Sciences (Shirley, NY).

**Proximate Analysis**

Fat, protein, and solids concentrations (g/100 g of milk) of milks were measured by a Fourier-transform mid-infrared milk analyzer (LactoScope FTIR, Delta Instruments BC, Drachten, the Netherlands) to verify formulation and for process control. Pre-calibration (Lynch et al., 2006) and calibration (Kaylegian et al., 2006) of the mid-infrared milk analyzer with modified milks were performed as described. Milk pasteurization was confirmed by alkaline phosphatase test (AOAC method 946.03, AOAC International, 2000; Phos-kit, Weber Scientific, Hamilton, NJ). Fat particle size was measured using a Mastersizer 2000 (Malvern Instrument Ltd., Malvern, UK) as described by Di Marzo et al. (2016) to confirm appropriate homogenization.
Descriptive Sensory Analysis

Sensory analysis was conducted in accordance with the North Carolina State University Institutional Review Board for the Protection of Human Subjects in Research regulations. Sensory properties of milk were evaluated by 6 trained panelists at d 3, 7, and 14. Each panelist (4 females, 2 males, ages 21 to 55 yr) had a minimum of 80 h of experience evaluating flavor and texture attributes of foods using the spectrum method (Meilgaard et al., 2007), and at least 40 h of experience with evaluation of fluid milk sensory attributes using an established sensory language (Croissant et al., 2007; Lee et al., 2017; McCarthy et al., 2017). Milks (30 mL) were dispensed into 59-mL soufflé cups with lids (Dart Container Corp., Mason, MI) with random 3-digit blinding codes. Samples were prepared with overhead lights off to prevent light oxidation, and tempered to 10°C. Each panelist evaluated each milk in duplicate using Compusense Cloud (Compusense, Guelph, Canada).

Extraction of Volatile Compounds

Headspace-SPME-GC-MS. Volatile compounds in milks were evaluated at d 0, 3, 7, and 14. All injections were made on an Agilent 7820 GC with 5975 MSD (Agilent Technologies Inc., Santa Clara, CA) with a ZB-5ms (30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex, Torrance, CA) column. Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (CTC Analytics, Zwingen, Switzerland). Analytical conditions for GC-MS were adapted from Vazquez-Landaverde et al. (2006). Five milliliters of milk along with 20 µL of internal standard (2-methyl-3-heptanone in ethyl ether at 81 mg/kg) was added to 20-mL SPME autosampler vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA) in triplicate. Vials were equilibrated for 25 min at 35°C with 4 s of
pulsed 250 rpm agitation. A single 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; Supelco, Bellefonte, PA) 1-cm fiber was used for all analysis. The SPME fiber was exposed to the samples for 40 min at a depth of 31 mm. The fiber was retracted and injected at 50 mm in the GC inlet for 5 min. The GC oven was initially held at 35°C for 3 min with a gradual increase of 10°C/min to 150°C and held for 1 min then raised at a rate of 20°C/min to 250°C and maintained for 5 min. The SPME fiber was introduced into the inlet with splitless mode at 250°C (0.75 min valve delay). All analyses were performed at a constant flow rate of 1 mL/min with helium. Scanning parameters were set from 30 to 350 m/z to identify compounds of interest. The MS transfer line was maintained at 250°C with the quad at 150°C and source at 230°C.

**Headspace-SPME-GC-O.** The SPME followed by GC-O for aroma active compound characterization was conducted on all milks (HTST, DSI-UP, and IND-UP) at d 0, 3, 7, and 14. All injections were made on an Agilent 6850 GC (Agilent Technologies Inc.) with ZB-5 and ZB-Wax (30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex) columns. Sample introduction was accomplished using a manual SPME holder (Supelco) equipped with an DVB/CAR/PDMS (Supelco) fiber. Ten milliliters of milk was added to 40mL amber vials. Vials were equilibrated for 30 min at 35°C, and the SPME fiber was exposed to the samples for 30 min at a depth of 20 mm. The fiber was retracted and injected at 30 mm in the GC inlet for 5 min. The GC oven was initially held at 35°C for 3 min with a gradual increase of 10°C/min to 150°C, then raised at a rate of 30°C/min to 250°C and maintained for 10 min. All analyses were performed at a constant flow rate of 2.0 mL/min with helium. Effluent was split 1:1 between the flame ionization detector and sniffing port using deactivated fused-silica capillaries (1 m length
The flame ionization detector sniffing port was held at a temperature of 300°C, and the port was supplied with humidified air at 30 mL/min. Each sample was evaluated on each column by at least 2 highly experienced sniffers (each with >50 h previous experience with GC-O) who recorded aroma character, perceived intensity, and retention time.

**SBSE-GC-O/MS.** Aroma active compounds from milks were also evaluated by SBSE-GC-O/MS. All injections were made on an Agilent 7890B GC with 5977 MSD attached with a ZB-5ms (30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex) column and an Olfactory Detection Port-3 (ODP-3) unit (Gerstel Inc., Mülheim an der Ruhr, Germany). Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (CTC Analytics). Thermal desorption was performed by a thermal desorption unit (Gerstel Inc.) in combination with a CIS-4 PTV injector (Gerstel). Sample preparation was modified from Hoffmann and Heiden (2000) and Park and Drake (2016) to include GC sniffing. Prior to analysis, the stir bars and thermal desorption unit tubes were conditioned for 1 h at 300°C. Ten milliliters of milk was placed in 10-mL amber vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical) along with a PDMS coated stir-bar (10 mm × 0.5 mm thickness, Gerstel) placed in the samples directly. The vials were stirred for 1 h at ambient temperature (21°C). The stir bars were washed with HPLC water (Fisher Chemicals, Fair Lawn, NJ), dried, and placed into clean thermal desorption tubes (Gerstel). Volatile compounds from the stir bars were thermally desorbed at 280°C assisted by cryofocusing at −100°C for 5 min. The GC inlet was then gradually increased to 275°C at 12°C/s, and held for 50 min. The GC oven was initially held at 35°C for 3 min with a rate of 10°C/min to 150°C, held for 1 min, then raised at a rate of
20°C/min to 250°C and maintained for 5 min. All analyses were performed at a constant flow rate of 1 mL/min with helium and the effluent from the capillary column was split 1:1 between the MS and the ODP-3. The ODP-3 exit was maintained at 250°C and humidified with air at 30 mL/min. Scanning parameters were set from 30 to 350 m/z. The MS transfer line was maintained at 280°C with the quad at 150°C and source at 230°C. Each sample was evaluated by at least 2 highly experienced sniffers (each with >50 h previous experience with GC-O) who recorded aroma character, perceived intensity, and retention time.

**Compound Identification and Quantification**

Aroma active compounds recovered from milks were identified by aroma properties, the National Institute of Standards and Technology (NIST, 2014) mass spectral database, authentic standards injection, and retention indices calculation (Vandendool and Kratz, 1963) using an alkane series (Sigma-Aldrich) under identical GC-O/MS conditions. Twenty-nine compounds, 7 of which were sulfur compounds, were selected for quantification based on aroma intensity and character, and previous literature (Colahan-Sederstrom and Peterson, 2005; Vazquez-Landaverde et al., 2006).

**HS-SPME-GC-MS/MS.** Quantification of selected compounds were made on an Agilent 7890B GC applied to an Agilent 7000C triple quad MS (MS/MS) and sulfur selective FPD (Agilent Technologies Inc.) equipped with a ZB-5ms (30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex) column. Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (CTC Analytics). Vials were equilibrated for 25 min at 35°C with 4 s of pulsed 250 rpm agitation. A single 50/30 um DVB/CAR/PDMS (Supelco) 1-cm fiber was used
for all analyses. The SPME fiber was exposed to the samples for 40 min at a depth of 31 mm. The fiber was retracted and injected at 50 mm in the GC inlet for 5 min. The GC oven was initially held at 35°C for 3 min with a gradual increase of 10°C/min to 150°C, held for 1 min, then raised at a rate of 20°C/min to 250°C and maintained for 5 min. The MS transfer line was maintained at 250°C with the quad at 150°C and source at 250°C. The flow rate of helium quench gas and nitrogen collision gas was 1.0 mL/min and 2.5 mL/min, respectively. Effluent from the capillary column was split 1:1 between the MS and FPD. The FPD for sulfur compounds was set to 325°C for transfer line, air 100 mL/min, and hydrogen 60 mL/min. The multiple reaction monitoring transitions and collision energies (CE) were developed to identify the most intensive/unique ions to the analytes of interest (Langen et al., 2013; Hopfer et al., 2016). The precursor ions for each compound were selected based on their mass spectra obtained from the full-scan single MS of authentic standards. The product ion scanning was acquired by GC-MS/MS using product ion scan mode with different CE of 3, 5, 10, 15, 20, 25, or 30 V with nitrogen gas. To obtain optimal ion abundances, selected compounds were tested again with 1, 2, 3, 4, and 5 V of CE based on compound volatility and molecular weight. The most intense product ion was chosen for the quantifier, and the second or third most intense product ions were selected for qualitative ions. Final multiple reaction monitoring transition was optimized with chosen CE to have optimal ion abundances for quantification (Table 2.1). Dwell times were set to ensure 3 to 3.1 cycles over a peak. MassHunter Qualitative and Quantitative analysis software (Agilent Technologies Inc.) was used for data analysis. External standard curves for 29 selected compounds were constructed using milk (skim HTST) as the matrix. Each standard stock solution was prepared, then diluted at either 1:2, 1:5, 1:10, 1:20, or 1:50 strength and the skim milk was then spiked with 20 µL of each concentration level. In addition to the milk and
standard, 2 internal standards (20 µL of ethyl methyl sulfide in ethyl ether at 1.65 mg/kg and 20 µL of 2-methyl3-heptanone in ethyl ether at 81 mg/kg) were also added to each 20-mL amber SPME vial. The response ratio of the quantifier ion to that of internal standard was calculated. All samples were injected using SPME fiber in triplicate at each concentration level, and linear regression plots were used to determine the calibration equations.

**Statistical Analyses**

The GLM procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) was used to determine the effect of fat level (0.2 and 2% fat milk), heat treatment (HTST, DSI-UP, and IND-UP), and time of storage (3, 7, and 14 d at 4°C) on descriptive sensory analysis attribute scores. The time variable (continuous) was a mean-centered (Misawa et al., 2016) variable to avoid colinearity effects on statistical analysis (Glantz and Slinker, 2001) and named as TimeT in models. Time was transformed by subtracting the mean time from each of the individual time and using these mean-centered data in the statistical analysis to provide a better point of reference in parsing the relative strength of main effects and interaction effects of factors in the model. If the F-value for the full model was significant ($P < 0.05$), then significance ($P < 0.05$) of each factor and their interactions was determined. The category effects of fat level, heat, replicate, and panelist and their interactions were tested for significance using the interaction term of fat × heat × replicate × panelist, whereas the linear effects of the continuous variable of time and interactions of the categorical variables were tested for significance using the full model error. The analysis for all factors and their interactions was done first, if the F-value for the full model was < 0.05, then a stepwise process was done to remove all nonsignificant terms from the model to produce a final reduced model and a type III sum of squares table was produced and the $R^2$ for the reduced
model was reported. Concentrations of selected compounds were analyzed by ANOVA (fat content × storage time x heat treatment) with means separation using Fisher’s least significant difference test. Principal component analysis was applied to visualize and differentiate aroma active compounds between milk treatments. All statistical analyses were performed using XLSTAT (version 2017, Addinsoft, New York, NY) at a significance of $P < 0.05$.

Results and Discussion

**Proximate Analysis**

Skim milk composition was $0.068\% \pm 0.004$ fat, $4.72\% \pm 0.02$ lactose, $3.12\% \pm 0.01$ protein, and $8.95\% \pm 0.03$ total solids (TS). The 2% fat milks averaged $1.96\% \pm 0.004$ fat, $4.63\% \pm 0.02$ lactose, $3.06\% \pm 0.01$ protein, and $10.7\% \pm 0.03$ TS. Milk composition was not different between treatments at each fat level ($P > 0.05$). The particle size distribution $d(0.9)$ [90% of the total fat globule volume in the sample comes from particles with diameter that lies below the $d(0.9)$] was < 1.5 $\mu$m for all milks, indicating appropriate homogenization. No differences ($P > 0.05$) were observed in milk fat particle size among the 2% milk treatments [mean $d(0.9)$ for 2% milk; HTST = 1.26, DSI-UP = 1.18, and IND-UP = 1.37 $\mu$m], which were in the range of particle size distribution [range of $d(0.9)$ from 1.19 to 1.81 $\mu$m] reported by Caplan and Barbano (2013). All milks were negative for alkaline phosphatase, indicating complete pasteurization.

**Descriptive Sensory Analysis**

Trained panel profiling of milks demonstrated that the milks were differentiated by overall aroma intensity, sweet aromatic, cooked, and sulfur/eggy flavors among heat treatments and across time (Tables 2.2 and 2.3 and Figure 2.1). Fat level (skim vs. 2% fat) had only a minor
direct influence on most trained panel perception of flavor, except for milk fat flavor (Table 2.2), as would be expected. Where fat did have an effect, it was manifested as an interaction effect in combination with heat treatment (Table 2.2) for overall aroma intensity and sweet aromatic, sulfur, and astringent flavors. Fat level did not affect overall aroma intensity ($P > 0.05$), but did affect ($P < 0.05$) all other sensory attributes (Tables 2.2 and 2.3), but the magnitude of the effect of fat was very small compared with heat treatment as seen from the very small proportion of the total type III sum of square explained by fat terms in the ANOVA model. The HTST milks had lower ($P < 0.05$) scores for all sensory attributes except sweet taste (Table 2.3). The UP milks treated by DSI or IND were distinguished by higher ($P < 0.05$) aroma intensity, cooked and sulfur/eggy flavors, and astringency compared with HTST milk (Table 2.3). The 2 UP treatments also were distinct in sensory profile. Milk processed by DSI-UP had higher overall aroma intensity and sulfur/eggy flavor (Table 2.3), whereas IND-UP milks had a higher ($P < 0.05$) intensity of sweet aromatic flavors. Time of storage had the largest effect ($P < 0.05$) on overall aroma intensity and sulfur flavor score with these scores decreasing with time of storage (Figure 2.1). There was an interaction ($P < 0.05$) of time of storage and heat treatment (Table 2.2) with overall aroma intensity decreasing more with time for UP milks than HTST milk and IND-UP milk decreasing in sulfur intensity more with time than DSI-UP milk (Figure 2.1).

Distinct cooked and sulfur/eggy flavors in milk have been reported as characteristic of UP/UHT milk due to the excessive heat applied compared with HTST (Liem et al., 2016; Lee et al., 2017). Sulfur-containing compounds contributing to the cooked and sulfur flavors are attributed to denaturation of serum protein, mainly β-lg and sulfur-containing amino acids (AA) in the serum protein fraction (Hutton and Patton, 1952; Mehta, 1980; AlAttabi et al., 2009). Cysteine, present at high amounts in serum protein, is known as a key precursor of hydrogen
sulfide and carbon disulfide under severe heat treatment (Al-Attabi et al., 2009). These compounds are known as major sulfur compounds responsible for cooked and sulfur flavors (Vazquez-Landaverde et al., 2005, 2006). The difference in the intensity of sulfur/eggy flavor between DSI-UP and IND-UP could be attributed to interactions between flavor compounds and serum protein. Denatured unfolded protein contains hydrophobic pockets, which result in hydrophobic interactions and binding with flavor compounds (Reineccius, 2006). Although IND-UP had higher serum protein denaturation than DSI-UP (Lee et al., 2017), the less intense sulfur flavor by IND-UP would be expected due to the extent of flavor binding in relation to the degree of protein denaturation.

Higher sweet aromatic flavor in IND-UP milk (Table 2.3) may be related to the effect of the timetemperature profile of DSI-UP, IND-UP, and HTST (Lee et al., 2017). Lee et al. (2017) documented that IND-UP generated a higher total heat exposure of the milk than DSI-UP, which was evaluated by furosine content. The higher furosine content of IND-UP suggests greater Maillard reactions and lactose degradation, which may contribute to sweet aromatic flavors. Potent sweet aromatic volatile compounds include maltol, 2-acetyl-1-pyrroline, furaneol, and sotolon in heated milk (Scanlan et al., 1968; Calvo and Hoz, 1992; Colahan-Sederstrom and Peterson, 2005). Decreases in aroma and flavor intensity of milks during storage was observed, consistent with previous studies (Chapman et al., 2001; Lee et al., 2017). However, UP milks remained distinct from HTST milks. Dissipated aroma and flavor during milk storage in our study (Figure 2.1) could be associated with diffusion of highly volatile compounds or oxidation of sulfur compounds or lipids (Al-Attabi et al., 2009).
Volatile Compound Analysis

Fifty-five and 45 aroma active compounds were detected in milks by SPME-GC-O and SBSE-GC-O, respectively. As expected, some highly volatile compounds (e.g., sulfur-containing compounds) were detected by SPME-GC-O and not by SBSE-GC-O (Table 2.4). In contrast, some higher molecular weight aroma active compounds (e.g., acids and lactones) identified by SBSE-GC-O were either tentatively identified or not detected by SPME-GC-O. Generally, SPME collects volatile compounds from headspace with a bias toward more highly volatile compounds (Drake et al., 2010; Smith et al., 2016). Headspace compounds play a major role in aroma perception, but may not represent comprehensive aroma profiles (Hoffmann and Heiden, 2000). On the other hand, SBSE was more effective to profile volatiles with heavy molecular weight or semivolatile compounds of milk, especially when applied with the multidimensional techniques of olfactometry and MS. The combination of the 2 extraction methods provided an effective platform to evaluate aroma active volatile and semi-volatile compounds and define/screen key flavor compounds, which span a wide range in milk. The majority of the aroma active compounds characterized in this study were previously identified in UP or UHT milk, but 17 compounds were newly detected in this study (Table 2.4). Among those 17 compounds, 12 compounds were positively identified and remaining 5 compounds were tentatively identified by either SPME or SBSE. Based on the frequency of detection among the 6 milks, odor characteristics, and literature (ColahanSederstrom and Peterson, 2005; Vazquez-Landaverde et al., 2006), 29 potent aroma active compounds were selected for quantification.

The combination of GC-MS followed by GC-MS/MS techniques used in this study extended the number of chemical class in one analytical run, unlike applying solvent extraction methods to cover multi-classes or high molecular weight volatile compounds.
(ColahanSederstrom and Peterson, 2005; Potineni and Peterson, 2005; Czerny and Schieberle, 2007). Confirmation of compounds was obtained by the ratio of 2 selected ions, one for quantification and one for qualification, which provided selective and sensitive response throughout MS/MS transition. In this study, optimization of MS/MS was shown to be a key tool to integrate different classes of target compounds in milk including sulfur, ketones, aldehydes, free fatty acid, lactones, and furanones. This wide range of analyzable compounds has not been previously reported using the GC-MS/MS technique in the field of dairy flavors.

Concentrations of selected aroma active compounds were distinct between UP and HTST milks across the storage days evaluated (Table 2.5). Regression coefficients for all calibration curves were > 0.95. Consistent with sensory profile, skim and 2% milks mainly differed by volatile compounds derived from lipids, such as hexanal, diacetyl, 2-heptanone, and lactones. The HTST milk had lower concentrations of sulfur compounds, ketones, and Strecker aldehydes compared with UP milks. This result would explain the lower intensity of overall aroma and lack of characteristic cooked and sulfur flavors in HTST milk. The UP milks contained higher concentrations of Maillard reaction compounds (2 and 3-methylbutanal, furfural, 2-acetyl-1-pyrroline, benzaldehyde), methyl ketones (2-butanone, 2-heptanone), and sulfur-containing compounds (hydrogen sulfide, carbon disulfide, dimethyl sulfide, and so on) compared with HTST, which contribute to the higher intensity of cooked and sulfur/eggy flavors of UP milk. Within UP treatments, hydrogen sulfide, dimethyl trisulfide, and methional were predominant in DSI-UP and 2 and 3-methylbutanal, diacetyl, furfural, 2-heptanone, 2-acetyl-1-pyrroline, 2-aminoacetophenone, benzaldehyde, and dimethyl sulfide were potent compounds in IND-UP.

The fat content of the milk affected the concentration of sulfur and various compounds related to Maillard reactions. Higher concentrations of furfural, 2-acetyl-1-pyrroline, 2-
aminoacetophenone, and benzaldehyde were present in 2% milks compared with skim, whereas higher concentrations of hydrogen sulfide and dimethyl sulfide were detected in skim milk ($P < 0.05$). This could be related to fat-flavor compound interactions (Reineccius, 2006). Some of these compounds, such as furfural, 2-acetyl-1-pyrroline, and 2-aminoacetophenone, have relatively low partition coefficients, making the compounds more soluble in water. As such, fat content may decrease retention in the matrix and cause release of more of these compounds into the headspace of 2% milks. On the other hand, hydrogen sulfide and dimethyl sulfide were more readily detected in skim milks compared with 2% milks possibly due to fat interference.

Christensen and Reineccius (1992) demonstrated that milk with higher fat content had a lower sensitivity to sulfur compounds by headspace analysis. Fat content can affect migration of some volatile compounds to the headspace (Belitz et al., 2009), and the concentration of flavor compounds and their perception (Drake et al., 2010; Yeh et al., 2017).

The fat level of milk also had an influence on the concentration of compounds derived from lipid regardless of the type of heat treatment, consistent with previous studies (Vazquez-Landaverde et al., 2005). Hexanal is derived from autoxidation of PUFA in milk and can be promoted via decomposition of hydroperoxides by heat (Grosch, 1982). Diacetyl is known as an important contributor to the buttery note in heated milk (Scanlan et al., 1968). Methyl ketones are naturally present in milk but their formation can be enhanced by milk fat degradation during heat treatment (Cadwallader and Singh, 2009), β-oxidation of saturated fatty acids followed by decarboxylation of β-ketoacids (Grosch, 1982). Higher concentration of 2-heptanone would be expected in 2% milk, because milk fat contains 10% of the fatty acids C6, 8, 10, and 12, which are precursors of odd-numbered carbon methyl ketones (C5, C7, C9, and C11; Vazquez-Landaverde et al., 2005). In addition, accelerated degradation of milk fat and hydroperoxides
during thermal treatment can result in the formation of methyl ketones (Cadwallader and Singh, 2009). Also, milk contains 1% lipids as oxo fatty acids, which can be liberated as β-ketoacids and decarboxylated to methyl ketones of C6–C16 when the milk fat is heated in the presence of water (Grosch, 1982). Lactones are formed by hydrolysis of hydroxyl acids from triglycerides (Reineccius, 2006). Hydroxy acids have no flavor as long as they are bound to triglycerides; the formation of lactones and flavor from fat are increased with heat treatment of milk (Reineccius, 2006; Drake et al., 2010). These compounds are known as thermal lipid degradation products in UP milk (Colahan-Sederstrom and Peterson, 2005), contributing milkfat and sweet coconut-like flavors to milk and dairy products (Drake et al., 2001).

Increases in compounds associated with Maillard reactions, including 2 and 3 methylbutanal, furfural, 2-acetyl-1-pyrroline, 2-aminoacetophenone, and benzaldehyde were higher in 2% UP milks than in HTST and skim milk, respectively. The concentration of these compounds was also higher in IND-UP compared with DSI-UP. Maillard reactions are one of the major pathways to develop flavors in milk and vary depending on the time-temperature profile (Reineccius, 2006). Because higher heat treatment can stimulate more Maillard reaction intermediates (Reineccius, 2006), more or higher concentrations of Maillard reaction compounds would be expected from IND-UP. A recent study by Lee et al. (2017) demonstrated that IND-UP was linked to elevated Maillard reactions between lactose and lysine residues of milk because the total heat load of IND-UP was higher compared with DSI-UP and HTST. In milk, Maillard reactions develop flavors associated with cooked and sweet aromatics, primarily under high heat (> 140°C; Belitz et al., 2009). Cooked flavor is affected by AA composition due to Maillard reactions being highly selective to the presence of specific AA to generate characteristic flavors. These AA include cysteine, lysine, methionine, leucine, isoleucine, valine, and tryptophan as
flavor precursors (Reineccius, 2006; Belitz et al., 2009). Two- and 3-methylbutanal are Strecker degradation products of branched-chain AA, isoleucine and leucine, respectively (Belitz et al., 2009). Previous studies indicated increased or accumulation of 2- and 3-methylbutanal along with the heat severity (Contarini and Povolo, 2002; Vazquez-Landaverde et al., 2005; Belitz et al., 2009). 2-Aminoacetophenone was identified in milk protein powders as a characteristic tortilla/ corn flavor (Smith et al., 2016). Smith et al. (2016) suggested that degradation of tryptophan by heat and alkali conditions would be the primary source of 2-aminoacetophenone in milk proteins. Belitz et al. (2009) indicated that aminoacetophenone can be formed from oxidation of skatole, which is derived from tryptophan. Because tryptophan content is higher in the serum protein fraction than casein (Belitz et al., 2009), the formation of 2-aminoacetophenone could be more related to serum denaturation and would be expected to be more prevalent in UP milks. Benzaldehyde is formed from phenylalanine (Belitz et al., 2009). Compounds derived from sugar degradation during Maillard reaction were predominant and are likely to increase sweet aromatic flavors in UP milks. Furfural is derived from sugars during heat processing, and it was previously reported as an indicator of heat treatment and a key compound of UP milk (Colahan-Sederstrom and Peterson, 2005; Vazquez-Landaverde et al., 2005). Furfural is known as the precursor of melanoidins in Maillard reactions in milk, which contribute to its darker color (BeMiller and Whistler, 1996). 2-Acetyl-1-pyrroline has a popcorn aroma, and was present at higher concentration in IND-UP, possibly imparting its higher sweet/cooked flavor. It is commonly found in many dairy products, such as milk proteins (Smith et al., 2016), dried milk powders (Karagul-Yuceer et al., 2001), and dried whey powders (Carunchia Whetstine et al., 2005), because 2-acetyl-1-pyrroline is formed in Strecker degradation of proline and readily formed even under mild heating (Reineccius, 2006; Belitz et al., 2009).
The effect of sulfur compounds on milk flavor was predominant in UP treatments. These compounds included hydrogen sulfide, carbon disulfide, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, and methional, similar with previous studies (Vazquez-Landaverde et al., 2006; Al-Attabi et al., 2009). Sulfur compounds have been associated with undesirable off-flavors, such as intense cooked and sulfur/eggy flavors in UP/UHT milk, especially with the IND method (Vazquez-Landaverde et al., 2006; Al-Attabi et al., 2009). However, to our knowledge, no studies have directly compared the sulfur compounds or flavor of UP milk between DSI and IND methods. Moreover, unlike previous studies which have demonstrated IND processing to develop higher cooked and sulfur flavors due to higher heat severity than DSI (Al-Attabi et al., 2009), we found distinct sulfur/eggy flavors and compounds in DSI-UP. This could be attributed to the differences of the heating methods and the time-temperature profiles between DSI and IND. In regard to distinct cooked and sulfur flavors between these 2 heating methods, it should be noted that DSI also may have more severe heat effect because of direct contact of steam and milk, which can maximize heat absorption. The IND-UP processing keeps the heating of the medium and milk separate, but overall showed a higher and longer total heat exposure. This fundamental difference could lead to a distinct profile of sulfur compounds between DSI and IND, shown by a significant increase of dimethyl sulfide in IND-UP, possibly due to further oxidation of other sulfur compounds. The IND-UP milk has a higher content of dissolved oxygen than DSI-UP milk, the latter process removes water, oxygen, and some highly volatile compounds in the vacuum chamber (Datta et al., 2002). In contrast, DSI-UP had higher dimethyl trisulfide than IND-UP, which might suggest a different degradation of methionine between these 2 heating methods possibly related to differences in oxygen level. Future studies should investigate the mechanisms of sulfur-containing AA between DSI-UP and IND-UP. The
primary source of sulfur compounds in milk can be generated by 2 reactions; Strecker
degradation of sulfur AA (cysteine/cystine and methionine), and liberation of sulfur compounds
by serum protein denaturation (Mehta, 2015). Although sulfur compounds are naturally present
in raw milk (Al-Attabi et al., 2009), dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, and
methional are formed from Maillard reactions of methionine, whereas hydrogen sulfide and
carbon disulfide can be derived from cysteine (Al-Attabi et al., 2009). Strecker degradation of
methionine forms methional, which is readily oxidized to dimethyl sulfide, and dimethyl
disulfide (Al-Attabi et al., 2009; Belitz et al., 2009). Methional was reported as a major off-
flavor marker of UHT milk (Colahan-Sederstrom and Peterson, 2005; Troise et al., 2014).
Dimethyl trisulfide is subsequently generated via oxidation of dimethyl sulfide where dimethyl
sulfoxide is formed as an intermediate compound (Al-Attabi et al., 2009). Dimethyl sulfoxide is
formed as an intermediate compound from oxidation of dimethyl sulfide (Shibamoto and Mihara,
1980), but its contribution to milk flavor is not confirmed yet.

Formation of hydrogen sulfide has been suggested in various ways: thermal denaturation
of β-lg and the sulfur-containing AA in serum proteins (Hutton and Patton, 1952), Strecker
degradation of cysteine and methionine (Belitz et al., 2009), and thermal degradation of thiamine
(Dwivedi and Arnold, 1973). Denaturation of individual serum proteins and degradation of sulfur
AA in serum protein have been hypothesized as primary sources of hydrogen sulfide in milk
because serum proteins are more sensitive to heat than casein (Mehta, 2015). More importantly,
cystine and cysteine are rich in serum protein, specifically β-lg, component of serum protein,
which can be key precursors of hydrogen sulfide during severe thermal processing (Al-Attabi et
al., 2009). In particular, the β-lg in serum protein contains 5 cysteine residues capable of forming
disulfide bonds as further flavor development (Mehta, 2015). β-Lactoglobulin is also more heat
labile than α-la and can be completely denatured by UP (Belitz et al., 2009). When β-lg is
denatured, the sulfhydryl groups readily release hydrogen sulfide and subsequently form
disulfide bonds or further reacts with other milk proteins (Mehta, 2015). Other possible sources
of formation of hydrogen sulfide in UP milks can be Strecker degradation of cysteine with
diketones or thermal degradation of thiamin via rearrangement of the thiazole group liberating
hydrogen sulfide (Al-Attabi et al., 2009). Future studies should identify the major source and
formation of sulfur compounds in UP milks.

Correlation analysis confirmed relationships between cooked and sulfur/eggy flavors and
the sulfur compounds hydrogen sulfide and carbon disulfide. Hydrogen sulfide was correlated
with sulfur/eggy flavor ($R^2 = 0.66; P < 0.05$) and carbon disulfide was correlated with cooked
flavor ($R^2 = 0.83; P < 0.05$), and sulfur/eggy flavor ($R^2 = 0.58; P < 0.05$). The correlation
coefficient of dimethyl sulfide, dimethyl disulfide, dimethyl sulfoxide, and dimethyl trisulfide for
cooked and sulfur/eggy flavors were under 0.50. Previous research suggested that hydrogen
sulfide and carbon disulfide have a linear relationship with either higher heat temperature or
cooked/heated flavor of milk (Hutton and Patton, 1952; Christensen and Reineccius, 1992;
Vazquez-Landaverde et al., 2006). Carbon disulfide has been an indicator of heat treatment
(Vazquez-Landaverde et al., 2006) and is a breakdown product of other sulfur compounds
(Urbach, 1993). The concentration of sulfhydryl groups decreases over time due to oxidation,
whereas that of disulfide bonds increases (Al-Attabi et al., 2009). These reactions could explain
the higher sensory intensity and concentration of sulfur compounds in freshly processed milks
and further the change of sulfur flavor and sulfur compound profiles over storage.
Relationship Between Volatile Compounds and Sensory Attributes

Principal component analysis was applied to visualize changes in flavor compound concentrations of milk processed with different heat treatments (Figures 2.2 and 2.3). The results revealed that most aroma active compounds were associated with UP milks by either DSI or IND at both fat levels, which pinpoints the distinct flavor differences between HTST and UP milks, and the relative lack of flavor of HTST milk. Comparing UP treatments, IND was more related to compounds derived from sugar degradation and Maillard reactions such as furfural, 2-acetyl-1-pyrroline, and maltol, which explains the higher sweet aromatic flavor in IND-UP milk. The IND-UP milks were also associated with sulfur compounds including dimethyl sulfoxide and dimethyl sulfide, possibly contributors to sulfur/eggy and cooked flavors. However, DSI-UP was more strongly associated with sulfurous compounds such as hydrogen sulfide, dimethyl trisulfide, and methional, which likely affect sulfur and cooked flavors in DSI-UP. Differentiation of sulfur compounds between UP treatments suggests that different AA precursors may be involved in the different heating mechanisms between IND and DSI, leading to distinct breakdown products and the distinct sensory profiles. Increased dimethyl sulfide in IND-UP may indicate further oxidation of methionine/dimethyl disulfide due to longer heating and cooling times (Lee et al., 2017). Dimethyl trisulfide in DSI-UP suggests Strecker degradation of methionine to methional and its oxidation to dimethyl trisulfide. Future work should investigate the reaction pathways of sulfur AA present in milk protein as a precursor of sulfur compounds and their role in UP milk.
Conclusions

The flavor of milk processed by HTST, DSI-UP, or IND-UP was characterized by descriptive sensory and instrumental analyses. Heat treatments and fat level had distinct effects on sensory profiles of milk as demonstrated by higher overall aroma, cooked, and sulfur/eggy flavors with UP milks compared with HTST milks. The DSI-UP and IND-UP differed from each other by sulfur/eggy, sweet aromatic, and cooked flavor intensities. The presence of Maillard reaction compounds (2 and 3-methylbutanal, furfural, 2-acetyl-1-pyrroline, benzaldehyde), methyl ketones (2-butanone, 2-heptanone), and sulfur-containing compounds (hydrogen sulfide, carbon disulfide, dimethyl sulfide, dimethyl trisulfide, methional) also distinguished UP milk from HTST, which were associated with the distinct cooked and sulfur/eggy flavors in UP milks. The major aroma active compounds were hydrogen sulfide, dimethyl trisulfide, and methional in DSI-UP and 2- and 3-methylbutanal, furfural, 2-heptanone, 2-acetyl-1-pyrroline, 2-aminoacetophenone, benzaldehyde, dimethyl sulfide in IND-UP. These findings demonstrate differences in flavor formation by Maillard reaction and protein denaturation during thermal processing that affect the flavor quality of UP milks and distinct sensory and chemical differences between DSI-UP and IND-UP milks.

Acknowledgments

This study was supported in part by the National Dairy Council (Rosemont, IL). The use of tradenames does not imply endorsement or lack of endorsement by those not mentioned.
REFERENCES


Table 2.1. Multiple reaction monitoring (MRM) transition parameters for selected compounds for gas chromatography-triple quadrupole mass spectrometry (GC-MS/MS) analytical conditions

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Precursor ion (m/z)</th>
<th>Quantifier ion (m/z)</th>
<th>CE&lt;sup&gt;1&lt;/sup&gt; (V)</th>
<th>Qualifier 1 ion (m/z)</th>
<th>CE (V)</th>
<th>Qualifier 2 ion (m/z)</th>
<th>CE (V)</th>
<th>Calibration curve</th>
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<tbody>
<tr>
<td>1</td>
<td>Hydrogen sulfide</td>
<td>34</td>
<td>32</td>
<td>3</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>2</td>
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<td>47</td>
<td>4</td>
<td>46</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td>Carbon disulfide</td>
<td>76</td>
<td>44</td>
<td>3</td>
<td>46</td>
<td>3</td>
<td></td>
<td></td>
<td>y = 15.305 x 0.99</td>
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<tr>
<td>4</td>
<td>2-Butanone</td>
<td>72</td>
<td>43</td>
<td>3</td>
<td>57</td>
<td>1</td>
<td></td>
<td></td>
<td>y = 0.0126 x 0.99</td>
</tr>
<tr>
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<td>Diacetyl</td>
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<td>43</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
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<td>3-Methylbutanal</td>
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<td>58</td>
<td>3</td>
<td>57</td>
<td>10</td>
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<td>10</td>
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<td>61</td>
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<td>48</td>
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<td>y = 0.0544 x 0.95</td>
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<td>2-Methylbutanal</td>
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<td>58</td>
<td>3</td>
<td>57</td>
<td>10</td>
<td>44</td>
<td>10</td>
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<tr>
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<td>Dimethyl disulfide</td>
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<td>Hexanal</td>
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<td>43</td>
<td>4</td>
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<td>Furfural</td>
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<td>40</td>
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<td>2-Heptanone</td>
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<td>15</td>
<td>83</td>
<td>5</td>
<td>69</td>
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<td>Methional</td>
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<td>76</td>
<td>1</td>
<td>48</td>
<td>4</td>
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<td>2-Methyl-3-heptanone</td>
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<td>85</td>
<td>3</td>
<td>86</td>
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<td></td>
<td>71</td>
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<td>1-Octen-3-one</td>
<td>97</td>
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<td>15</td>
<td>69</td>
<td>10</td>
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<td>Dimethyl trisulfide</td>
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<td>80</td>
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<td>5</td>
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<td>1-Octen-3-ol</td>
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<td>3</td>
<td>43</td>
<td>3</td>
<td></td>
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<td>18</td>
<td>Butyric acid</td>
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<td>55</td>
<td>10</td>
<td></td>
<td></td>
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<tr>
<td>19</td>
<td>Benzaldehyde</td>
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<td>10</td>
<td>51</td>
<td>10</td>
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<tr>
<td>20</td>
<td>Phenyl acetate</td>
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<td>94</td>
<td>10</td>
<td>66</td>
<td>20</td>
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<td>Furaneol</td>
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<td>57</td>
<td>10</td>
<td></td>
<td></td>
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<tr>
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<td>71</td>
<td>15</td>
<td>97</td>
<td>10</td>
<td>55</td>
<td>15</td>
<td>y = 7.7084 x 0.96</td>
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Table 1. Continued

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<th>No.</th>
<th>Compound</th>
<th>Precursor ion (m/z)</th>
<th>Quantifier CE (V)</th>
<th>Product ion Qualifier 1 CE (V)</th>
<th>Product ion Qualifier 2 CE (V)</th>
<th>Calibration curve</th>
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<tr>
<td>23</td>
<td>2-Acetyl-2-thiazoline</td>
<td>129</td>
<td>60</td>
<td>5</td>
<td>101</td>
<td>y = 1.9929 x</td>
</tr>
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<td></td>
<td></td>
<td>0.98</td>
</tr>
<tr>
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<td>Sotolon</td>
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<td>82</td>
<td>4</td>
<td>100</td>
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<td>2-Aminoacetophenone</td>
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<td>120</td>
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<td></td>
<td></td>
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<td>0.98</td>
</tr>
<tr>
<td>26</td>
<td>δ-Octalactone</td>
<td>142</td>
<td>87</td>
<td>5</td>
<td>99</td>
<td>y = 6.7516 x</td>
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<td></td>
<td>0.99</td>
</tr>
<tr>
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<td>Skatole</td>
<td>130</td>
<td>77</td>
<td>15</td>
<td>103</td>
<td>y = 2.9212 x</td>
</tr>
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<td></td>
<td>0.97</td>
</tr>
<tr>
<td>28</td>
<td>γ-Decalactone</td>
<td>170</td>
<td>95</td>
<td>15</td>
<td>85</td>
<td>y = 9.6237 x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.99</td>
</tr>
<tr>
<td>29</td>
<td>δ-Decalactone</td>
<td>152</td>
<td>97</td>
<td>3</td>
<td>84</td>
<td>y = 9.6237 x</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.99</td>
</tr>
</tbody>
</table>

1 CE = collision energy.
2 ISTD = internal standard.
Table 2.2. ANOVA type III sum of squares for descriptive sensory profiles of HTST and ultrapasteurized (UP) skim and 2% fat milks at d 3, 7, and 14 post processing at 4°C

<table>
<thead>
<tr>
<th>Factor 1</th>
<th>Sensory attributes 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall aroma</td>
</tr>
<tr>
<td>Fat</td>
<td>0.00</td>
</tr>
<tr>
<td>Heat</td>
<td>311.47*</td>
</tr>
<tr>
<td>Replicate</td>
<td>0.02</td>
</tr>
<tr>
<td>Panelist</td>
<td>0.09</td>
</tr>
<tr>
<td>Fat x heat</td>
<td>4.91*</td>
</tr>
<tr>
<td>Fat x rep</td>
<td>NS</td>
</tr>
<tr>
<td>Heat x rep</td>
<td>NS</td>
</tr>
<tr>
<td>Fat x heat x rep x panelist</td>
<td>5.73</td>
</tr>
<tr>
<td>TimeT</td>
<td>67.13*</td>
</tr>
<tr>
<td>TimeT x fat</td>
<td>NS</td>
</tr>
<tr>
<td>TimeT x heat</td>
<td>11.18*</td>
</tr>
<tr>
<td>TimeT x rep</td>
<td>NS</td>
</tr>
<tr>
<td>r-squared</td>
<td>0.912</td>
</tr>
</tbody>
</table>

1 Heat had 3 levels (HTST = high temperature short time; DSI-UP = ultrapasteurization by direct steam injection; IND-UP = ultrapasteurization by indirect heating); fat has 3 levels skim = milk with 0.2% fat and 2% = milk with 2% fat; Replicate has 3 levels, replicate 1, 2, and 3; TimeT is the mean centered time with 3 levels, 3, 7, and 14 days.
2 NS = model term not significant (P > 0.05).
* P < 0.05.
Table 2.3. Least square means for descriptive sensory profiles of skim and 2% fat milks with different thermal treatments (HTST and ultrapasteurized (UP) milks) stored at d 3, 7, and 14 post processing at 4°C

<table>
<thead>
<tr>
<th>Factor</th>
<th>Treatment</th>
<th>Sensory attributes$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Overall aroma</td>
</tr>
<tr>
<td>Fat</td>
<td>Skim</td>
<td>2.3$^a$</td>
</tr>
<tr>
<td>2%</td>
<td></td>
<td>2.3$^a$</td>
</tr>
<tr>
<td>Heat</td>
<td>HTST</td>
<td>1.5$^c$</td>
</tr>
<tr>
<td></td>
<td>DSI-UP</td>
<td>2.9$^a$</td>
</tr>
<tr>
<td></td>
<td>IND-UP</td>
<td>2.4$^b$</td>
</tr>
</tbody>
</table>

1 Attribute intensities were scored on a 0 to 15-point universal intensity scale (Meilgaard et al., 2007). Fluid milk flavors fall between 0 and 4 on this scale (Drake, 2007; Croissant et al., 2007; McCarthy et al., 2017).

2 HTST = high temperature short time; DSI-UP = ultrapasteurization by direct steam injection; IND-UP = ultrapasteurization by indirect heating; skim = milk with 0.2% fat and 2% = milk with 2% fat.

3 0.0 = not detected.

a, b, c Least square means within a column are different within fat level and heat treatment if they do not share a common superscript ($P < 0.05$).
Table 2.4. Aroma active compounds of milk detected by gas chromatography-olfactometry (GC-O) using headspace solid-phase microextraction (HS-SPME) and stir bar sorptive extraction (SBSE)

<table>
<thead>
<tr>
<th>RI^1</th>
<th>Compound</th>
<th>Odor Description^2</th>
<th>Identification^3</th>
<th>Previously identified in UP or UHT milk^5,^6</th>
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</thead>
<tbody>
<tr>
<td>ZB-5</td>
<td>Wax</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>544</td>
<td>Hydrogen sulfide</td>
<td>Sulfur/egg</td>
<td>O,RI,MS</td>
<td>ND^4</td>
</tr>
<tr>
<td>564</td>
<td>Dimethyl sulfide</td>
<td>Chemical/sulfur</td>
<td>O,RI,MS</td>
<td>ND^4</td>
</tr>
<tr>
<td>568</td>
<td>Carbon disulfide</td>
<td>Cooked</td>
<td>O,RI,MS</td>
<td>ND^4</td>
</tr>
<tr>
<td>596</td>
<td>2-Butanone</td>
<td>Plastic</td>
<td>O,RI,MS</td>
<td>ND^4</td>
</tr>
<tr>
<td>607</td>
<td>Diacetyl</td>
<td>Diacetyl</td>
<td>O,RI,MS</td>
<td>ND^4</td>
</tr>
<tr>
<td>645</td>
<td>2-Methylbutanal</td>
<td>Cooked/malty</td>
<td>O,RI,MS</td>
<td>ND^4</td>
</tr>
<tr>
<td>665</td>
<td>3-Methylbutanal</td>
<td>Cooked/malty</td>
<td>O,RI,MS</td>
<td>O,RI,MS</td>
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<tr>
<td>701</td>
<td>Acetoin</td>
<td>Sweet/milky</td>
<td>O,RI,MS</td>
<td>ND^4</td>
</tr>
<tr>
<td>752</td>
<td>Dimethyl disulfide</td>
<td>Earthy/sulfur</td>
<td>O,RI,MS</td>
<td>ND^4</td>
</tr>
<tr>
<td>767</td>
<td>Dimethyl sulfoxide</td>
<td>Sulfur/garlic</td>
<td>O,RI,MS</td>
<td>ND^4</td>
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<tr>
<td>785</td>
<td>2-Hexanone</td>
<td>Metallic/burnt</td>
<td>O,RI</td>
<td>ND^4</td>
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<tr>
<td>808</td>
<td>Hexanal</td>
<td>Grass</td>
<td>O,RI,MS</td>
<td>O,RI,MS</td>
</tr>
<tr>
<td>823</td>
<td>Butanoic acid</td>
<td>Acidic/cheesy</td>
<td>O,RI,MS</td>
<td>O,RI,MS</td>
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<tr>
<td>831</td>
<td>Methyl pyrazine</td>
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<td>O,RI</td>
<td>ND^4</td>
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<tr>
<td>857</td>
<td>Furfural</td>
<td>Barny/brothy</td>
<td>O,RI,MS</td>
<td>O,RI,MS</td>
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<td>Ethyl 2-methylbutanoate</td>
<td>Fruity</td>
<td>O,RI</td>
<td>ND^4</td>
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<tr>
<td>861</td>
<td>Furfuryl alcohol</td>
<td>Burnt/woody</td>
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<tr>
<td>867</td>
<td>Isovaleric acid</td>
<td>Sweaty/sour</td>
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<td>ND^4</td>
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<tr>
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<td>Sulfur/nutty/meaty</td>
<td>O,RI</td>
<td>ND^4</td>
</tr>
<tr>
<td>897</td>
<td>2-Heptanone</td>
<td>Cooked</td>
<td>O,RI,MS</td>
<td>O,RI,MS</td>
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<tr>
<td>898</td>
<td>Heptanal</td>
<td>Earthy/fatty</td>
<td>O,RI,MS</td>
<td>O,RI,MS</td>
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<tr>
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<td>Ethyl valerate</td>
<td>Fruity</td>
<td>O,RI,MS</td>
<td>ND^4</td>
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<tr>
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<td>O,RI,MS</td>
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<td>Popcorn/cereal</td>
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<td>determination methods</td>
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<td>-----------------------</td>
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</tr>
<tr>
<td>944</td>
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<td>Benzaldehyde</td>
<td>Cooked/nutty</td>
<td>O,RI,MS O,RI,MS</td>
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<td>Feed</td>
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<td>961</td>
<td>1151</td>
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<td>Sweet/fruity</td>
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<tr>
<td>984</td>
<td>1369</td>
<td>Dimethyl trisulfide</td>
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<td>990</td>
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<tr>
<td>1147</td>
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<td>(E)-2-nonenal</td>
<td>Oily</td>
<td>O,RI O,RI,MS 11</td>
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<td>1155</td>
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<td>(E,Z)-2,6-nonadienal</td>
<td>Herb/nutty</td>
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<td>Decanal</td>
<td>Soapy</td>
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<td>Phenylacetic acid</td>
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<td>Bread/sweet</td>
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<td>Acidic/sweaty</td>
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<tr>
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<td>(E,E)-2,4-decadienial</td>
<td>Fatty</td>
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<tr>
<td>1354</td>
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<td>2-Aminoacetophenone</td>
<td>Tortilla/grain</td>
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<td>Decanoic acid</td>
<td>Cosmetic/soapy</td>
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<td>Fecal/foul</td>
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<tr>
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<td>Vanillin</td>
<td>Vanilla</td>
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<tr>
<th>RI(^1)</th>
<th>Compound</th>
<th>Odor Description(^2)</th>
<th>Identification(^3)</th>
<th>Previously identified in UP or UHT milk(^5, 6)</th>
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<tr>
<td>ZB-5</td>
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<td>Wax</td>
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<tr>
<td>1486</td>
<td>γ-Decalactone</td>
<td>Sweet/caramel</td>
<td>ND</td>
<td>O, RI, MS</td>
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<td>δ-Decalactone</td>
<td>Fruity</td>
<td>ND</td>
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</tr>
<tr>
<td>1553</td>
<td>δ-Undecalactone</td>
<td>Butter sweet</td>
<td>O, RI, MS</td>
<td>2, 6, 7, 11</td>
</tr>
<tr>
<td>1593</td>
<td>Dodecanoic acid</td>
<td>Soapy</td>
<td>O, RI, MS</td>
<td>O, RI, MS</td>
</tr>
<tr>
<td>1649</td>
<td>γ-Dodecalactone</td>
<td>Sweet/grain</td>
<td>O, RI, MS</td>
<td></td>
</tr>
<tr>
<td>1693</td>
<td>2-Pentadecanone</td>
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<td>ND</td>
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<td>1753</td>
<td>Tetradecanoic acid</td>
<td>Baby powder</td>
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<tr>
<td>1879</td>
<td>δ-Dodecalactone</td>
<td>Sweet/grain</td>
<td>O, RI, MS</td>
<td>O, RI, MS</td>
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</table>

1 Retention indices (RI) calculated from GC-O on the ZB-5 and Wax columns with SPME, and ZB-5 column with SBSE.
2 Odor description at the GC sniffing port by SPME and SBSE.
3 Aroma active compounds identification from SPME and SBSE by O = comparison of the odor description at the sniffing port to the reference; RI = retention index; MS = mass spectrum obtained by gas chromatography-mass spectrometry.
4 ND = not detected.
5 The compounds were previously identified in milk treated with ultrapasteurized (UP) or ultra-high temperature (UHT) by the authors given in parenthesis; 1 (Vazquez-Landaverde et al., 2006); 2 (Belitz et al., 2009); 3 (Valero et al., 2001); 4 (Vazquez-Landaverde et al., 2005); 5 (Potineni and Peterson, 2005); 6 (Moid et al., 1994); 7 (Czerny and Schieberle, 2007); 8 (Marsili, 1999); 9 (Kokkinidou and Peterson, 2014); 10 (Jeon et al., 1978); 11 (Colahan-Senderstrom and Peterson, 2005); 12 (Troise et al., 2014); 13 (Contarini et al., 1997); 14 (Mestdagh et al., 2005); 15 (Scanlan et al., 1968); 16 (Al-Attabi et al., 2014); 17 (Simon et al., 2001).
6 Literature numbers 1-8 = UHT milk purchased from retail (heating type not addressed); 9-12 = UHT milk processed in lab or pilot scale (heating type not addressed); 13 = UHT milk processed by direct steam injection; 14-16 = UHT milk processed by indirect heating; 17 = UP milk processed by indirect heating.
Table 2.5. Concentration (µg/kg) of selected aroma active compounds in milk

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<tr>
<th>Compound</th>
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<tr>
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<td>Day 0</td>
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</tr>
<tr>
<td>H2S</td>
<td>8.1</td>
<td>16.9</td>
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<tr>
<td></td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>DMS</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>CS2</td>
<td>1.5</td>
<td>7.0</td>
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<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>DMDS</td>
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<td>2.3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.6</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Methional</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DMTS</td>
<td>1.4</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Ketones</td>
<td></td>
<td></td>
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<tr>
<td>2-butanone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diacetyl</td>
<td>2.1</td>
<td>1.2</td>
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1 Sulfur compounds
2 H2S1
3 DMS1
4 CS21
5 DMDS1
6 DMSO1
7 Methional
8 DMTS1
9 Ketones
10 2-butanone
11 Diacetyl

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Table 2.5. Continued

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<thead>
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<th>Compound</th>
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<td>Skim</td>
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<td></td>
<td>Day 0</td>
</tr>
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<tr>
<td>2-heptanone</td>
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<td>Aldehydes</td>
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<td>1-octen-3-one</td>
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137
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<tr>
<td>Butyric acid</td>
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<td>Furanones and Lactones</td>
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<tr>
<td>Maltol</td>
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<tr>
<td>Butyric acid</td>
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<tr>
<td>Phenyl acetate</td>
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138
Table 2.5. Continued

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<td>Day 0</td>
<td>Day 3</td>
</tr>
<tr>
<td>HTST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IND</td>
<td></td>
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</tbody>
</table>

| HTST | DSI | IND | HTST | DSI | IND | HTST | DSI | IND | HTST | DSI | IND | HTST | DSI | IND | HTST | DSI | IND | HTST | DSI | IND | HTST | DSI | IND | HTST | DSI | IND | HTST | DSI | IND |
|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|
| 10.5 | 16  | 8.6 | 12.6 | 11.5| 8.9 | 12.1 | 10.7| 9.26| 10.7 | 11.6| 9.93| 8.58 | 10.6| 10.0| 14.5 | 12.7| 11.9| 10.7 | 13.0| 9.6 | 15.9 | 12.9| 0.15|
| 12.2 | 12  | 7.8 | 11.7 | 12.8| 8.0 | 12.0 | 15.7| 15.2| 14.8 | 13.4| 11.6| 16.8 | 13.7| 11.1| 11.8 | 15.1| 12.1| 9.76 | 16.9| 12.8| 8.5 | 13.3 | 13.4| 0.18|

1 H$_2$S = hydrogen sulfide; DMS = dimethyl sulfide; CS$_2$ = carbon disulfide; DMDS = dimethyl disulfide; DMSO = dimethyl sulfoxide; DMTS = dimethyl trisulfide; 3MB = 3-methylbutanal; 2MB = 2-methylbutanal; 2A1P = 2-acetyl-1-pyrroline
2 HTST = high temperature short time; DSI = ultra-pasteurization by direct steam injection; IND = ultrapasteurization by indirect heating.
3 Means within a row that differ by the LSD are different within fat level, heat treatment and days of storage time (P < 0.05).
Figure 2.1. Trained panel intensities for overall aroma and sulfur/eggy flavor with different heat treatment and storage. HTST = high temperature short time; DSI = ultrapasteurization by direct steam injection; IND = ultrapasteurization by indirect heating.
Figure 2.2. Principal component biplot for aroma active compound concentration of skim milks across 14 days storage at 4°C. HTST = high temperature short time; DSI = ultrapasteurization by direct steam injection; IND = ultrapasteurization by indirect heating.
Figure 2.3. Principal component biplot for aroma active compound concentration of 2% milks across 14 days storage at 4°C. HTST = high temperature short time; DSI = ultrapasteurization by direct steam injection; IND = ultrapasteurization by indirect heating.
CHAPTER 3: IDENTIFICATION OF THE SOURCE OF VOLATILE SULFUR COMPOUNDS IN MILK DURING THERMAL PROCESSING

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The content of this chapter is submitted for publication in:

Journal of Dairy Science
Abstract

The presence of volatile sulfur compounds in ultrapasteurized (UP) milk are the major contributors to sulfur/burnt and eggy flavors and these flavors are disliked by consumers. Previous research has established distinct differences in flavor profiles of fluid milk processed by high temperature short time pasteurization (HTST) and UP by direct steam injection (DSI-UP) or indirect heat (IND-UP). An understanding of the contribution of the individual milk proteins to sulfur off-flavors would clarify the source of sulfur flavors in UP milks. The objective of this study was to determine the source of volatile sulfur compounds in fluid milk with a specific focus on the comparison of heat treatment effects on milks by HTST and UP. Reformulated skim milks (RSM) were manufactured by blending micellar casein concentrate (MCC) and serum protein isolate (SPI) at three different ratios (MCC:SPI; 95:5, 80:20, and 60:40) as a percent of total protein (3.3%) to determine the source of sulfur/burnt and eggy flavors. Freshly processed MCC or SPI at equivalent protein content as skim milk (3.3%) were blended with milk permeate and lactose. Raw skim milk served as a control. Skim milk and RSM were pasteurized at 78°C for 15s (HTST) or 140°C for 2.3s by IND-UP or DSI-UP. The experiment was replicated three times. Sensory properties of milks and RSM were documented by descriptive sensory analysis. Volatile sulfur compounds in milks and RSM were evaluated using solid phase micro-extraction (SPME) followed by gas chromatography-triple quadrupole mass spectrometry (GC-MS/MS) combined with a sulfur selective flame photometric detector. Sensory panelists confirmed increased overall aroma, cooked, sulfur/burnt and eggy flavors in skim milk and RSM processed by DSI-UP followed by IND-UP and HTST. RSM with higher SPI as a percent of total protein had higher sulfur/burnt and eggy flavors along with elevated concentrations of hydrogen sulfide and carbon disulfide compared to skim milk or RSM with lower SPI ratios. Sulfur compounds
including dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, dimethyl sulfoxide and methional were not associated with sulfur/burnt and eggy flavors which suggests that these compounds may not specifically contribute to the sulfur/burnt and eggy off-flavors of UP milks. The combination of sensory and instrumental methods used in the current study effectively identified the source of sulfur compounds in milk, and further confirmed the contribution of hydrogen sulfide and carbon disulfide to eggy and sulfur/burnt flavors, respectively. These results demonstrate and confirm that serum protein is the source of sulfur/burnt and eggy off-flavors in UP milk and provide baseline information for flavor of fluid milk and milk protein-based beverages that require UP.

**Key words:** fluid milk, volatile sulfur compounds, ultrapasteurization
Introduction

Thermal processing of milk has facilitated product safety and a longer shelf life. In the United States, milk processing involves high temperature short time (HTST) pasteurization (minimum of 72°C for 15 s), providing ca 3 wk of shelf life, and ultrapasteurization (UP) and/or ultrahigh-temperature (UHT) are for extended shelf life of milk (Boor, 2001). The thermal processing conditions for UP milk are defined as at or above 138°C (280°F) for at least two (2) seconds (21 CFR 131.3, FDA, 2017), and this product generally has an extended shelf life (ESL) under refrigerated conditions, up to 3 months. UHT milk is the same thermal process as for UP milk, but the milk is aseptically packaged to be shelf stable (PMO, FDA, 2015).

Despite the advantage of a longer shelf life compared to conventional HTST milk, UP and UHT milk have stronger overall flavor intensities, including undesirable cooked and sulfur/eggy off-flavors which can create a possible barrier to UP/UHT milk acceptance by consumers (Perkins and Deeth, 2001; Lee et al., 2017). Recently, Jo et al. (2018) demonstrated that sulfur compounds, including hydrogen sulfide (H₂S), carbon disulfide (CS₂), dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS), distinguished UP milks from HTST milks. These compounds were associated with the higher cooked flavor and distinct sulfur/eggy flavors in UP milks. The study further suggested that H₂S and CS₂ were the key responsible compounds for sulfur/eggy flavor. Jo et al. (2018) also established that the sulfur compound profile was different between the two heat treatment methods for UP milk, indirect heating (IND) and direct steam injection (DSI). Those key compounds included H₂S, DMTS, and methional for DSI-UP and DMS for IND-UP, indicating that differences in flavor formation by Maillard reactions and protein denaturation during thermal processing affected the flavor quality.
of UP milks and resulted in distinct flavor differences between DSI-UP and IND-UP milks (Jo et al., 2018).

The primary source of volatile sulfur compounds in milk has been suggested to be due to the denaturation of serum (whey) protein and sulfur containing amino acids in serum protein (Hutton and Patton, 1952), as well as the Maillard reaction, a thermally induced reaction between sulfur containing amino acids and reducing sugars (McGorrin, 2011). The amount of sulfur containing amino acids (cystine/cysteine, methionine) in milk are variable based on the milk protein fraction; casein and serum. Methionine is present in casein and serum protein at equivalent levels (Belitz et al., 2009), which possibly can make both casein and serum proteins as the primary source of DMS, DMDS, and DMTS via Strecker degradation. Cystine and cysteine are higher in serum protein than casein, which can be key precursors of H$_2$S and CS$_2$ during heat treatment of milk (Belitz et al., 2009). Constituents of serum protein including β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA) and immunoglobulin (Ig) contain sulfur amino acids in different ratios. β-lg has a higher amount of methionine, and α-la contains higher amount of cystine/cysteine (Heine et al., 1991). Changes in properties of these milk proteins are affected by heat treatment (temperature), leading to denaturation, which involves unfolding and subsequent aggregation of unfolded protein molecules and flavor formation (Kühn et al., 2006). As serum protein is more heat labile than casein (Brodkorb et al., 2013), the effect of heat treatment on serum protein can be more significant than casein in relation to distinct sulfur/cooked flavors.

There are established characteristic properties of serum protein in response to the heat treatment of milk. Those include the content of sulfhydryl groups (Hutton and Patton, 1952; Pofahl and Vakaleris, 1968), and the denaturation of serum protein and β-lg (Hutton and Patton,
1952; Larson and Rolleri, 1955; Lyster, 1970; Manji and Kakuda, 1986; Elliott et al., 2005; Lee et al., 2017). Although studies have suggested that these properties are the source(s) of the formation of sulfur compounds and sulfur and cooked flavors (Hutton and Patton, 1952; Mehta, 1980; Calvo and de la Hoz, 1992), previous studies have not elucidated the protein fractions and the compounds responsible for the distinct sulfur flavor in milk in conjunction with different heat treatments. A greater understanding of the source(s) of cooked and sulfur flavors in UP milk can be obtained if flavor properties of milk protein fractions generated during milk heat treatment are elucidated.

Membrane technology has been applied in milk and dairy products since the 1970s and has greatly developed dairy processing as a number of unit operations involve water or bacteria removal, and milk fat or protein separation (Pouliot, 2008). Membrane separation of milk proteins has expanded the versatility of dairy ingredients and facilitated the development of new concepts of dairy products, such as ESL milks, and high protein beverages (Smithers, 2008; Pouliot, 2008). The application of microfiltration (MF) to skim milk followed by ultrafiltration (UF) can be applied to generate serum protein isolate (SPI) and 95% serum protein reduced micellar casein concentrate (MCC) (Zulewska et al., 2009; Beckman et al., 2010; Nelson and Barbano, 2005). The formation of sulfur compounds can be dependent on these distinct protein fractions and their physical changes caused by heat treatment. More importantly, identification of the source of sulfur compounds from milk proteins during thermal processing of milk can help to indicate sulfur off-flavors in UP milk and further predict consumer acceptability. No previous studies have compared protein source and impact of heat treatment on milk and skim milk models processed by HTST or UP. The objective of this study was to determine the source of volatile sulfur compounds in fluid milk using skim milk and reformulated skim milk (RSM) with
distinct milk protein fractions and to elucidate the impact of thermal process on sulfur flavor compound formation.

Materials and Methods

Skim Milk and Reformulated Skim Milk (RSM) Production

Source of skim milk and RSM. Raw bovine skim milk (0.087% ± 0.003 fat, somatic cell count <300,000) was obtained from the North Carolina State University Dairy Enterprise System (Raleigh, NC). The raw skim milk was divided into two portions: one for skim milk, and one for MCC and SPI production. This process was replicated twice on two different days.

MCC Manufacture. Raw skim milk (530 kg) was HTST pasteurized at 72°C for 15 s with a plate heat exchanger (model T4 RGS-16/2, SPX Flow Technology, Greensboro, NC) and cooled to 4°C. The pasteurized skim milk was prefiltred by a Nexis T filter (NXT 10-30U-M7S, Pall Corp., Port Washington, NY). The milk was then heated to 50°C with a plate heat exchanger (serial no. G201400849, Plate ID: SR1, SPX Flow Technology). The pasteurized and prefiltred skim milk was microfiltered using a ceramic MF system (Tetra Alcross M7, TetraPak Filtration Systems, Aarhus, Denmark), equipped with 0.1 µm nominal pore diameter graded permeability membrane (model EP1940GL0.1 u, AGP1020, alumina, Pall Corp.). A 3-stage and a continuous 3× MF process were used to produce a 95% serum protein reduced MCC with true protein (TP) concentration between 8.4 to 8.6% as described by Zulewska and Barbano (2014) and Cheng et al. (2018). Before the first stage, 160 kg of milk was taken into the MF system to flush water and both the retentate and permeate collected from this time were discarded. The first stage started when the flush ended. Then, 370 kg of milk was added as the feed for the first stage. In the
second and third stages, the MF retentate was diluted with DI water (2 kg of DI water for every 1 kg of retentate), heated to 50°C, and diafiltered with the ceramic MF system to produce 3× retentate. This diafiltration (DF) was repeated to complete a 3-stage process and the MF system was run continuously and not stopped and restarted from stage to stage. At the end of stage 3, all retentate (liquid MCC) was mixed, cooled to 4°C, transferred into light shielded HDPE half-gallon containers (Upstate Niagara Cooperative, Buffalo, NY), and stored at 4°C and then used for RSM production the following day.

**SPI Manufacture.** Several batches of SPI were produced and combined to obtain enough liquid SPI for the study (Cheng et al., 2018). Raw skim milk was HTST pasteurized at 72°C for 15 s and prefiltered as previously described. The MF permeate collected from the first stage of MCC production was placed in the UF feed tank and heated to 50°C with a plate heat exchanger. The UF feed was processed by a 2-stage UF and the UF retentate was used to produce liquid SPI as described by Cheng et al. (2018). The UF process was continued until the protein concentration in the UF retentate reached between 27 to 28% protein measured by a Fourier-transform mid-infrared milk analyzer (LactoScope FTIR; Delta Instruments BC, Drachten, the Netherlands). The final UF retentate (liquid SPI) from the second stage was transferred into light shielded HDPE half-gallon containers (Upstate Niagara Cooperative) and stored at -18°C until RSM production.

**RSM Production.** RSM was prepared by blending three different ratios of MCC (95%, 80%, and 60%) and SPI (5%, 20%, and 40%) as a percentage of the final protein content (3.3%) to simulate a skim milk model (Table 3.1). Final protein and lactose content of each RSM was
adjusted to the equivalent percentage as skim milk (3.3% and 4.7%, respectively) with each protein blend and with UF permeate from SPI production and lactose (Hilmar 5120 200 mesh, Hilmar Ingredients, Hilmar, CA).

**Thermal Processing**

Raw skim milk and blended RSM were processed by HTST, DSI-UP, or IND-UP as described by Lee et al. (2017). A microthermics EHVH pasteurization unit (Microthermics, Raleigh, NC) with a Microthermics Steam Injection Module and a 2-stage homogenizer (model NS2006H, GEA Niro Soavi, Parma, Italy) was used for milk processing. For the HTST treatment, raw skim milk and blended RSM was preheated to 60°C, pasteurized at 78°C for 15 s and cooled to 10°C. The DSI-UP products were preheated to 90°C, pasteurized at 140°C for 2.3 s under 330 kPa pressure by direct culinary steam injection (model LG-30, Electro-Steam Generator Corp., Alexandria, VA) using a Microthermics Steam Injection Module with Cub 5 software (version 3.1), then cooled to 85°C by vacuum cooling under a 1,040-mmHg vacuum. The DSI-UP milk was then homogenized and cooled to 10°C. For the IND-UP, products were preheated to 90°C, then UP at 140°C for 2.3 s by the indirect heater. The milk was cooled to 85°C before homogenization and then cooled by a second cooler to 10°C. All milks were homogenized at 20.7 MPa total pressure with 3.4 MPa on the second stage. All milks were packaged in light shielded, half-gallon HDPE containers (Upstate Niagara Cooperative), stored at 4°C, and sampled at d 1 post processing for analyses.
Chemical Composition

Fat, protein, and lactose concentration (g/100 g of milk) of milks and RSM were measured by a mid-infrared milk analyzer to verify formulation and process control compared to raw skim milk. Pre-calibration (Lynch et al., 2006) and calibration (Kaylegian et al., 2006) of the mid-infrared milk analyzer with modified milks were performed as described (Wojciechowski et al., 2016). Milk pasteurization was confirmed by alkaline phosphatase test (AOAC method 946.03; Phos-kit, Weber Scientific, Hamilton, NJ). All pasteurized milks were tested for aerobic plate count (APC; AOAC International, 2012; method 990.12) and coliform count (AOAC International, 2012; method 991.14) (Petrifilm, 3M, St. Paul, MN) to evaluate microbial quality.

Descriptive Sensory Analysis

Sensory analysis was conducted in accordance with the North Carolina State University Institutional Review Board for the Protection of Human Subjects in Research regulations. Sensory properties of milk and RSM were evaluated by 7 trained panelists at d 1 post processing. Each panelist (4 females, 3 males, ages 21 to 55 y) had a minimum of 80 h of experience evaluating flavor and texture attributes of dairy products using the SpectrumTM method (Meilgaard et al., 2007), and at least 40 h of experience with evaluation of fluid milk sensory attributes using an established sensory language (Croissant et al., 2007; McCarthy et al., 2017; Lee et al., 2017, Jo et al., 2018). One addition to the current study was that sulfur/eggy flavor was split into two distinct aromatics: sulfur/burnt flavor (burnt/cooked flavor not eggy in nature) and eggy flavor (sulfur aromatic like hard boiled egg), to further clarify the nature of the specific cooked flavors that were only encountered in UP milk. Milks (30 mL) were dispensed into 59 mL soufflé cups with lids (Dart Container Corp., Mason, MI) with random 3-digit blinding.
codes. Samples were prepared with overhead lights off to prevent light oxidation, and tempered to 10°C. Each panelist evaluated each milk in duplicate using Compusense Cloud (Compusense, Guelph, Canada).

**Volatile Sulfur Compounds Analysis**

**Headspace-SPME-GC-MS/MS.** Selected sulfur compounds including hydrogen sulfide (H₂S), carbon disulfide (CS₂), dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl sulfoxide (DMSO), methional, and dimethyl trisulfide (DMTS) were measured using an Agilent 7890B gas chromatograph applied to an Agilent 7000C triple quad mass spectroscopy (GC-MS/MS) and sulfur selective flame photometric detector (FPD; Agilent Technologies Inc., Santa Clara, CA) equipped with a ZB-5ms column (30 m length x 0.25 mm i.d. x 0.25 µm film thickness; Phenomenex, Torrance, CA). Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (CTC Analytics, Zwingen, Switzerland). Five (5) ml of milk or RSM along with 20 µl of internal standard (ethyl methyl sulfide in ethyl ether at 1.65 mg/kg; Sigma Aldrich, St. Louis, MO) was added to 20 ml SPME autosampler vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA). The analytical conditions and the multiple reaction monitoring (MRM) transition for selected sulfur compounds (Table 3.2) were followed as described by Jo et al. (2018). Dwell times were set to ensure 3-3.1 cycles over a peak. The experiments were performed in triplicate. MassHunter Qualitative and Quantitative Analysis software (Agilent Technologies Inc.) were used for data analysis. The relative concentration of each compound was calculated based on the response ratio of the quantifier ion to that of internal standard.
**Orthonasal Threshold Analysis**

H₂S and CS₂ were selected for orthonasal threshold testing to align with the volatile compound analysis and sensory intensities of sulfur/burnt and eggy flavors in UP milk. A modification of the American Society for Testing and Materials (ASTM) procedure E679–9 (ASTM, 2004), an ascending forced choice (AFC) method of limits, was used to determine best estimate orthonasal threshold values for each compound as a 7-series 3-AFC. Stock solutions of each compound were prepared in 95% ethyl alcohol (Sigma-Aldrich). Aliquots of the stock solutions were placed into the selected medium (skim milk, HTST pasteurized at 77°C for 25 s). HTST skim milk for threshold testing was obtained from North Carolina State University Dairy Enterprise System within 1 d of post processing. The concentrations of these two compounds in HTST skim milk are below the LOQ and this milk has no discernable sulfur/eggy flavor (Jo et al., 2018). Blank solutions were skim milk with no added compound. The orthonasal detection thresholds of each of each compound were determined individually. Stock solutions were serially diluted with the respective diluent (step factor of 1.5), and 30 mL of each was poured into clean, 3-digit-coded 60 mL lidded soufflé cups (Dart Container Corp.). The samples for each threshold test were prepared 3 h before the test to achieve equilibrium for each compound in the sample cup (Leksrisompong et al., 2010). Subjects (n = 38) were instructed on the appropriate sniffing procedure before testing as described by Leksrisompong et al. (2010). Seven ascending series were presented with sample presentation randomized within each series. A 1-min rest was enforced between each set of 3 samples. Responses were collected using paper ballots.
Descriptive Analysis of Model Systems with Sulfur Compounds

Skim milk model systems containing spiked additions of targeted sulfur compounds were prepared to further confirm specific contributions to eggy and sulfur/burnt flavors of UP milk. H2S and CS2 were selected as primary contributors to the eggy and sulfur/burnt flavors, respectively. Stock solutions of each compound were prepared individually in 95% ethyl alcohol (Sigma-Aldrich) and spiked into HTST pasteurized skim milk (77°C for 25 s) at the range of orthonasal threshold and average concentration documented in UP milk (Jo et al., 2018). A control with no added compounds was also prepared. Eggy and sulfur/burnt flavor intensities were documented in model systems in duplicate by a trained panel as described previously. The experiment was replicated twice.

Statistical Analyses

The data from the experiment with RSM and different heat treatment were analyzed by 2-way analysis of variance (ANOVA) (heat treatment x protein ratio) to determine the effect of serum protein ratio in RSM (skim milk, RSM 1, RSM 2, RSM 3) and heat treatment (HTST, DSI-UP, IND-UP) with means separation using Fisher’s least significant difference (LSD) test. For threshold results, the individual best estimate threshold (BET) was taken as the geometric mean of the last concentration with an incorrect response and the first concentration with a correct response, except for the following sequence: if the subject indicated a “not sure” response for the correct choice, that concentration was increased by a factor of 1.41 to adjust for the possibility of a chance correct response (Lawless et al., 2000). The group BET was taken as the geometric mean of the individual BET values. Sensory results from model systems were
evaluated by ANOVA with Fisher’s LSD test. All statistical analyses were performed using XLSTAT (version 2018, Addinsoft, New York, NY) at $P < 0.05$ significance.

**Results and Discussion**

**Chemical Composition**

Skim milk composition was 0.074% ± 0.005 fat, 4.62% ± 0.04 lactose, 3.29% ± 0.02 protein, and 9.07% ± 0.06 total solids. The RSM averaged 0.049% ± 0.008 fat, 4.65% ± 0.12 lactose, 3.06% ± 0.03 protein, and 8.78% ± 0.03 total solids. RSM compositions were not different between treatments at each protein ratio ($P > 0.05$). All milks were negative for alkaline phosphatase, indicating complete pasteurization. No coliforms were detected in pasteurized skim milks and RSM. All milks had APC less than $10^2$ CFU/mL.

**Descriptive Sensory Analysis**

Trained panel profiling of milks and RSMs demonstrated that the milks were different in overall aroma intensity, sweet aromatic, cooked/milky, sulfur/burnt and eggy flavors and astringency by both protein ratio and heat treatment ($P < 0.05$) (Table 3.3). As expected, UP treatments by DSI or IND were distinguished by higher overall aroma, cooked/milky flavor and astringency compared to HTST in all skim milk and RSM ($P < 0.05$). Regardless of SPI content in RSM, HTST products had no changes in cooked/milky flavor and no detectable sulfur/burnt or eggy flavors. This result indicated that the development of sulfur/burnt and eggy flavors were due to UP heat treatment, in agreement with previous studies of UP milk flavors (Lee et al., 2017; Jo et al., 2018). Astringency was increased in all UP milk and RSM compared to HTST products but was also distinct within the UP treatments and was higher in RSM with increased
serum protein ratios. Higher astringency in UP treatments compared to HTST was consistent with previous studies (Lee et al., 2017; Liem et al., 2016). Josephson et al. (1967) reported a higher astringency in whey than skim milk in a given heat treatment. The development of astringency in milk could be due to serum protein denaturation and aggregation with UP heat treatment (Josephson et al., 1967; Beecher et al., 2008). Hutton and Josephson (1951) suggested that β-lg was presumed to be a significant contributor to the astringency in heat treated milk. In addition, the order of homogenization and thermal process may have an influence on astringency of milk and milk protein-based beverages (Lee et al., 2017). Different ratios of milk protein fractions (MCC and SPI) affected other flavors of the RSM processed with UP ($P < 0.05$, Table 3.3). Cooked/milky flavor increased with heat treatment and SPI ratio. Higher serum protein content RSM had higher intensities of cooked/milky flavor in both UP treatments, DSI and IND compared to HTST. The development of cooked flavor in UP milk has been suggested to be derived from milk protein denaturation, particularly serum protein, possibly in relation to the formation of significant amounts of volatile sulfur compounds (Hutton and Patton, 1952; Patrick and Swaisgood, 1976; Mehta, 1980; Calvo and Hoz, 1992).

Sulfur/eggy flavor was previously documented in studies with UP milk. Lee et al. (2017) and Jo et al. (2018) documented distinct differences in the sensory profiles, especially sulfur/eggy flavor intensity of DSI-UP and IND-UP skim and 2% milk fat milks. In the current study, this flavor was split into two distinct aromatics: sulfur/burnt flavor (burnt/cooked flavor not eggy in nature) and eggy flavor (sulfur aromatic like hard boiled egg), in an effort to clarify the nature of these cooked flavors that were only encountered in UP milk. This process could also help clarify flavor differences generated from each heat treatment (DSI vs. IND). The distinct differences in the sensory profiles of DSI and IND in previous studies were the
intensities of sulfur/eggy and sweet aromatic flavors. In the current study, DSI-UP skim had a lower sweet aromatic flavor than IND-UP skim, consistent with previous studies (Table 3.3). Cooked/milky, sulfur/burnt and eggy flavors were not distinct between DSI-UP skim and IND-UP skim, but DSI and IND RSM with 20 or 40% SPI as a proportion of the protein had distinct eggy flavor intensities that were higher than those in the RSMs with 5% SPI as a proportion of the protein ($P < 0.05$). DSI RSMs also had higher intensities of sulfur/burnt flavor than IND RSMs. The differences in the intensities of sulfur/burnt and eggy flavors could be associated with specific milk protein denaturation in relation to the differences in heat treatment. The DSI process can denature serum proteins in a short time period due to the rapid temperature increase, whereas serum protein denaturation during the IND process can take a longer period of time (Oldfield et al., 1998; Lee et al., 2017). This difference may impact the formation of sulfur/burnt and eggy flavors in UP milks.

The differences in these 2 flavors (sulfur/burnt and eggy) between DSI and IND were not significant when skim milks were compared. Although RSM 2 had equivalent protein ratios to skim milk, the sulfur/burnt and eggy flavors were more distinct in RSM 2 than skim milk, possibly due to the recombination of milk protein fractions which could increase available sulphydryl groups at the time of heat treatments (Adams et al., 2001). Moreover, it is possible that the properties of the β-lg and casein interactions in milk are not the same as those interactions formed in isolated systems of serum protein and casein (Sawyer, 1969). The interaction between serum protein and casein when milk or individual milk proteins are heated can influence the flavor character of milk, which can also be important to the applications of milk proteins. In addition, the relative rates of these interactions would be dependent on temperature and heating profile of the heat treatment (Oldfield et al., 1998), which can affect the
relative rates of unfolding and the formation of sulfur compounds. Future studies should investigate the interaction between thermally processed milk proteins and flavor compounds in relation to the flavor development or retention of flavors of milk-based beverages.

It has been recognized that cooked and sulfur flavors (sulfur/burnt and eggy) in UP milks are formed by milk proteins during heat treatments, but the direct evidence to the source of off-flavors has not been addressed. Previous studies investigated structural, functional, and physical changes of individual milk protein fractions (casein and serum) following heat treatment (Hutton and Patton, 1952; Jenness, 1954; Oldfield et al., 1998; Anema and Li, 2003), however, it is difficult to determine the off-flavor sources in milk without understanding the sensory and flavor chemistry perspective. The skim milk models used in the current study provide clarification of sensory attributes of milk and milk protein fractions as well as how processing conditions impact flavor formation in UP milk.

**Volatile Compound Analysis**

Concentrations of volatile sulfur compounds, except DMSO, were distinct in skim milk and RSMs across different heat treatments \((P < 0.05, \text{Table 3.4})\). The concentration of H\(_2\)S and CS\(_2\) increased in RSM with increased SPI ratio \((P < 0.05, \text{Table 3.4})\). Volatile sulfur compound profiles also differed by UP treatment; DSI or IND. DSI-UP had higher concentration of CS\(_2\), DMTS, methional and DMSO, while IND-UP was higher in DMS. This observation can be directly linked to the fact that differences in heating mechanisms affect the formation of sulfur compounds. Higher concentrations of DMS in IND-UP would be expected as IND-UP milk contains a higher level of dissolved oxygen and requires longer cooling times, which can lead to further oxidation of other sulfur compounds including methional and DMDS (Datta et al., 2002;
Jo et al., 2018). The current results are consistent with a previous study, which reported higher amounts of DMS in IND-UP milk compared to DSI-UP milk (Jo et al., 2018). Methional is a high impact aroma compound in UP and UHT milk (Jo et al., 2018; Colahan-Sederstrom and Peterson, 2005), derived from Strecker degradation of methionine (Belitz et al., 2009). Higher concentrations of H\textsubscript{2}S and CS\textsubscript{2} were attributed to both UP treatments compared to HTST treatments. These compounds were reported as the main sulfur compounds in UP milks contributing to sulfur/eggy and cooked flavors (Jo et al., 2018). IND-UP milks had lower concentrations of CS\textsubscript{2} compared to DSI-UP. This could be explained by dissolved oxygen levels during the IND process. Extended heat treatment activates sulfhydryl groups in serum protein, which generates CS\textsubscript{2}, and makes them more oxidizable by atmospheric oxygen (Jenness, 1954). The reactive sulfhydryl group is more readily oxidized in liquid system including fluid milk (Jenness, 1954). The origin and formation of CS\textsubscript{2} in milk has not been clearly elucidated, and it is presumably generated from a breakdown product of other sulfur compounds (Urbach, 1993).

Volatile sulfur compounds were affected by serum protein ratio (Table 3.4). Changes in H\textsubscript{2}S and CS\textsubscript{2} were specifically attributed to higher serum protein content ($P < 0.05$). However, DMS, DMDS, DMTS and methional were not affected by serum protein ratio in RSM ($P > 0.05$). This may be related to the content of sulfur containing amino acids in milk protein fractions, casein and serum protein. Sulfur compounds including DMS, DMDS, and DMTS originate from the degradation of methionine (Pripis-Nicolau et al., 2000), one of the main sulfur containing amino acids in milk proteins. Serum protein and casein contain relatively equivalent amounts of methionine (Belitz et al., 2009). As such, changes in protein ratio did not impact amounts of methionine or compounds purportedly sourced to methionine.
The increase of \( \text{H}_2\text{S} \) and \( \text{CS}_2 \) in RSM with higher SPI as a proportion of total protein can be linked to the higher amount of cysteine in serum protein (Belitz et al., 2009). The elevated levels of these sulfur compounds, especially in RSM with higher SPI, directly support the assumption that serum protein is the source of sulfur/burnt and eggy off-flavors in milk treated with severe heat treatment such as UP, as those flavors were not detected in HTST milk and those compounds were not associated with HTST milk (Table 3.3, Figure 3.1). Studies reported that the degradation of cysteine alone produced the greatest amount of volatile compounds compared to other sulfur containing precursors (Zhang and Ho, 1991; Pripis-Nicolau et al., 2000). Pripis-Nicolau et al. (2000) noted that greater olfactory perception by cysteine was due to cysteine undergoes competitive reactions between Strecker degradation and decarboxylation, and releases sulfur compounds. As such, it is assumed that cysteine in serum protein could contribute to sulfur/burnt and eggy off-flavors more than methionine. This result further suggests that sulfur containing amino acids in serum protein play an important role in determination of the source of sulfur/burnt and eggy off-flavors in milks.

Consistent with the sensory results, the concentrations of some volatile sulfur compounds in skim milk were different from RSM 2, which had equivalent protein ratios to skim milk. \( \text{H}_2\text{S} \) and \( \text{CS}_2 \) in RSM 2 were at higher concentration than skim milk, which supports the higher sensory intensities of eggy flavor in RSM 2 by IND and DSI and higher sulfur/burnt intensity in RSM 2 by DSI compared to their respective skim milk counterparts. These significant differences were observed in UP treatments. It is possible that recombined milk proteins in RSM received more heat than native skim milk proteins, which can increase available sulphydryl groups (Adams et al., 2001).
**Orthonasal Threshold of Selected Sulfur Compounds**

Orthonasal detection BET values for $H_2S$ and $CS_2$ were calculated in skim milk (Table 3.5). The values were higher than published values in water. Threshold values for these compounds in milk matrix have not been previously reported. Although water and skim milk have similar pH, protein and/or fat content in milk affects thresholds (Hofmann et al., 2001). As such, it is critical to evaluate the threshold value of these compounds in skim milk because thresholds can vary considerably in different of matrices or by different methodology. The current study could expand the sensory and flavor perspective of sulfur/burnt and eggy flavors in milk since sensory threshold determination can be more significant than the concentration of the chemicals (Teranishi et al., 1991).

**Confirmation of Sulfur Compounds in Model System**

Following threshold determination of $H_2S$ and $CS_2$, HTST skim milk models spiked with those compounds were evaluated to verify specific eggy and sulfur/burnt flavors of UP milk (Table 3.6). The descriptive panel documented the eggy and sulfur/burnt flavors in the skim milk models containing $H_2S$ and $CS_2$, and the intensities of those specific attributes were similar to DSI-UP or IND-UP milks (Tables 3.3 and 3.6). The skim milk model system confirmed the contribution of $H_2S$ and $CS_2$ to characteristic eggy and sulfur/burnt flavors in milk.

**Relationship Between Volatile Sulfur Compounds and Sulfur Off-Flavors**

Partial least squares (PLS) regression further demonstrates the changes in volatile sulfur compound profiles in milks and RSMs and how they were affected by both heat treatment and the increase of serum protein as a percentage of total protein, which pinpoints the distinct eggy
and sulfur/burnt flavors and the higher concentration of H$_2$S and CS$_2$ (Figure 3.1). These flavors and compounds have been discussed in previous studies on fluid milk flavor as a general aspect and the possibilities of sulfur compounds contributing to the cooked and sulfur off-flavors in UP or UHT milks (Vazquez-Landaverde et al., 2006; Al-Attabi et al., 2009; Al-Attabi et al., 2014). However, these studies had insufficient information to conclude which specific compounds were responsible for specific sulfur off-flavors in UP or UHT milks. Since distinct eggy and sulfur/burnt flavors have been demonstrated as consumer defects for UP milk (Lee et al., 2017), a clear understanding of the nature and source of these flavors are crucial. The differentiation of DMS, DMDS, DMTS, DMSO and methional are likely derived from the differences in processing mechanisms between DSI and IND, such as heat transfer and dissolved oxygen during the heat process. These compounds were previously described as sulfur notes related to characteristic cabbage and vegetable odors in UP milk (Jo et al., 2018), which may not be directly linked to eggy and sulfur/burnt off-flavors encountered in the current study. These compounds can be possibly imparting overall aroma or cooked/milky flavor, however, no clear relationship were found by PLS or correlation analysis. A primary precursor of these compounds is known to be methionine, which is present in similar amounts in both casein and serum protein (Belitz et al., 2009). Increases in H$_2$S and CS$_2$ contribute to eggy and sulfur/burnt off-flavors in UP milk and originate from serum protein. Correlation analysis confirmed that eggy and sulfur/burnt flavors were correlated with H$_2$S ($R^2 = 0.94; P < 0.05$, $R^2 = 0.72; P < 0.05$) and CS$_2$ ($R^2 = 0.88; P < 0.05$, $R^2 = 0.70; P < 0.05$). The correlation coefficient for DMS, DMDS, DMTS, DMSO and methional for eggy and sulfur/burnt flavors were below 0.50 ($P < 0.05$), which along with their aroma properties, suggests that these compounds are not primary contributors to the eggy and sulfur/burnt off-flavors in UP milks. As such, the general trend of increased H$_2$S was
associated with eggy flavor and higher intensity of sulfur/burnt flavor could be attributed to increases in both H$_2$S and CS$_2$. Higher concentrations of both H$_2$S and CS$_2$ intensify sulfur flavors of UP milk, documented as sulfur/eggy flavor in previous studies.

**Conclusion**

The approach of manufacturing RSM by mixing MCC and SPI in different ratios allowed further clarification of the source of sulfur off-flavors in skim milk processed by UP. Heat treatment and serum protein ratio had significant effects on eggy and sulfur/burnt flavors and volatile sulfur compound profiles. Sensory and instrumental analysis results suggested that sulfur compounds including DMS, DMDS, DMTS, DMSO and methional originated from the differences in processing mechanisms between DSI and IND. Increased H$_2$S and CS$_2$ were correlated with distinct eggy and sulfur/burnt off-flavors derived from serum protein and attributed to UP treatment. A potential interaction between milk proteins and sulfur compounds affected by serum protein associated with casein during UP treatment was also documented. Orthonasal threshold calculations and descriptive analysis of model systems confirmed that H$_2$S and CS$_2$ were responsible for eggy and sulfur/burnt off-flavors, respectively. These results suggest that those compounds are critical indicators to determine sulfur off-flavors in UP milk. These findings also provide a practical insight into the source of heat generated sulfur flavors in UP milk as well as milk protein-based beverages that may require UP.

**Acknowledgements**

This study was supported in part by the National Dairy Council (Rosemont, IL). The use of tradenames does not imply endorsement or lack of endorsement by those not mentioned.
REFERENCES


Table 3.1. Reformulated skim milk (RSM) formulations

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Concentration (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>RSM 1</th>
<th>RSM 2</th>
<th>RSM 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCC&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>60</td>
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<td>20</td>
<td>40</td>
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</tr>
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<td>Lactose</td>
<td>4.7</td>
<td>4.7</td>
<td>4.7</td>
<td></td>
</tr>
</tbody>
</table>

1 % by w/w.
2 MCC = micellar casein concentration with 95% serum protein reduction. Concentration is presented by casein as a percentage of true protein (3.3%).
3 SPI = serum protein isolate. Concentration is presented by a percentage of true protein (3.3%).
Table 3.2. Multiple reaction monitoring (MRM) transition parameters for selected compounds for gas chromatography-triple quad mass spectrometry (GC-MS/MS) analytical conditions

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Precursor ion ((m/z))</th>
<th>Product ion</th>
<th>Quantifier ((m/z))</th>
<th>CE(^1) (V)</th>
<th>Qualifier ((m/z))</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hydrogen sulfide</td>
<td>34</td>
<td>32</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Dimethyl sulfide</td>
<td>62</td>
<td>47</td>
<td>4</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Carbon disulfide</td>
<td>76</td>
<td>44</td>
<td>3</td>
<td>46</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>ISTD</td>
<td>Ethyl methyl sulfide</td>
<td>76</td>
<td>61</td>
<td>2</td>
<td>48</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Dimethyl disulfide</td>
<td>94</td>
<td>79</td>
<td>10</td>
<td>64</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Dimethyl sulfoxide</td>
<td>78</td>
<td>63</td>
<td>3</td>
<td>61</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Methional</td>
<td>104</td>
<td>76</td>
<td>1</td>
<td>48</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Dimethyl trisulfide</td>
<td>126</td>
<td>80</td>
<td>15</td>
<td>61</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

1 CE = collision energy.
2 ISTD = internal standard.
Table 3.3. Descriptive sensory profiles of skim milks and reformulated skim milk (RSM) with different thermal treatments at d 1 post processing at 4°C

<table>
<thead>
<tr>
<th>Treatment²</th>
<th>Type²</th>
<th>Sensory attributes¹</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Overall aroma</td>
<td>Sweet</td>
<td>Cooked/</td>
<td>Sulfur</td>
<td>Eggy</td>
<td>Sweet</td>
<td>Astrin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>aromatic</td>
<td>milky</td>
<td></td>
<td></td>
<td>taste</td>
<td>gent</td>
</tr>
<tr>
<td>HTST</td>
<td>Skim</td>
<td>2.3 c</td>
<td>1.5 cde</td>
<td>3.3 d</td>
<td>ND</td>
<td>ND</td>
<td>2.1 a</td>
<td>2.0 d</td>
</tr>
<tr>
<td></td>
<td>RSM 1</td>
<td>1.6 d</td>
<td>1.7 bcd</td>
<td>3.0 d</td>
<td>ND</td>
<td>ND</td>
<td>2.0 a</td>
<td>2.0 d</td>
</tr>
<tr>
<td></td>
<td>RSM 2</td>
<td>1.8 cd</td>
<td>1.9 ab</td>
<td>3.1 d</td>
<td>ND</td>
<td>ND</td>
<td>2.1 a</td>
<td>2.0 d</td>
</tr>
<tr>
<td></td>
<td>RSM 3</td>
<td>1.6 d</td>
<td>1.6 bcd</td>
<td>3.0 d</td>
<td>ND</td>
<td>ND</td>
<td>2.0 a</td>
<td>2.2 cd</td>
</tr>
<tr>
<td>DSI-UP</td>
<td>Skim</td>
<td>3.5 b</td>
<td>1.1 ef</td>
<td>4.2 bc</td>
<td>1.5 b</td>
<td>1.5 c</td>
<td>2.0 a</td>
<td>3.4 a</td>
</tr>
<tr>
<td></td>
<td>RSM 1</td>
<td>2.0 cd</td>
<td>1.5 cde</td>
<td>3.9 c</td>
<td>0.8 c</td>
<td>ND</td>
<td>2.0 a</td>
<td>2.5 c</td>
</tr>
<tr>
<td></td>
<td>RSM 2</td>
<td>4.0 b</td>
<td>1.3 def</td>
<td>4.5 ab</td>
<td>2.0 a</td>
<td>2.5 b</td>
<td>2.0 a</td>
<td>3.0 ab</td>
</tr>
<tr>
<td></td>
<td>RSM 3</td>
<td>4.0 b</td>
<td>1.0 f</td>
<td>4.6 ab</td>
<td>2.3 a</td>
<td>3.1 a</td>
<td>2.0 a</td>
<td>3.2 a</td>
</tr>
<tr>
<td>IND-UP</td>
<td>Skim</td>
<td>3.5 b</td>
<td>1.8 bc</td>
<td>4.2 bc</td>
<td>1.8 b</td>
<td>1.8 c</td>
<td>2.0 a</td>
<td>3.0 ab</td>
</tr>
<tr>
<td></td>
<td>RSM 1</td>
<td>2.3 c</td>
<td>2.2 a</td>
<td>3.9 c</td>
<td>0.5 c</td>
<td>ND</td>
<td>2.0 a</td>
<td>2.7 bc</td>
</tr>
<tr>
<td></td>
<td>RSM 2</td>
<td>4.0 b</td>
<td>1.8 abc</td>
<td>4.5 ab</td>
<td>0.7 c</td>
<td>2.8 b</td>
<td>2.0 a</td>
<td>3.1 ab</td>
</tr>
<tr>
<td></td>
<td>RSM 3</td>
<td>4.8 a</td>
<td>1.7 bcd</td>
<td>4.7 a</td>
<td>0.9 c</td>
<td>3.5 a</td>
<td>2.0 a</td>
<td>3.4 a</td>
</tr>
</tbody>
</table>

| P value    | Heat      | <.0001              | <.0001 | <.0001 | <.0001 | <.0001 | 0.003 | <.0001 |
|            | Protein   | 0.003               | <.0001 | <.0001 | <.0001 | <.0001 | 0.008 | <.0001 |
|            | Heat x Protein | <.0001         | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |

1 Attribute intensities were scored on a 0 to 15-point universal intensity scale (Meilgaard et al., 2007). Fluid milk flavors fall between 0 and 4 on this scale (Drake, 2007; Croissant et al., 2007; McCarthy et al., 2017).

2 HTST = high temperature short time; DSI-UP = ultrapasteurization by direct steam injection; IND-UP = ultrapasteurization by indirect heating; skim = milk with 0.1% fat; RSM = reformulated skim milk blended with micellar casein concentrate (MCC) and serum protein isolate (SPI).

3 ND = not detected.

4 Means in the same column followed by a different superscript indicate significant ($P < 0.05$).
Table 3.4. Volatile sulfur compounds in skim milks and reformulated skim milk (RSM) with different thermal treatments at d 1 post processing at 4°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type</th>
<th>Hydrogen sulfide</th>
<th>Dimethyl sulfide</th>
<th>Carbon disulfide</th>
<th>Dimethyl disulfide</th>
<th>Dimethyl trisulfide</th>
<th>Methional</th>
<th>Dimethyl sulfoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTST</td>
<td>Skim</td>
<td>6.12 f</td>
<td>5.45 c</td>
<td>15.9 g</td>
<td>0.04 a</td>
<td>0.03 cd</td>
<td>0.30 c</td>
<td>0.002 a</td>
</tr>
<tr>
<td></td>
<td>RSM 1</td>
<td>4.89 f</td>
<td>5.37 c</td>
<td>13.1 g</td>
<td>0.01 bc</td>
<td>0.07 ab</td>
<td>0.63 bc</td>
<td>0.002 a</td>
</tr>
<tr>
<td></td>
<td>RSM 2</td>
<td>6.20 f</td>
<td>5.20 c</td>
<td>15.7 g</td>
<td>0.01 bc</td>
<td>0.05 bc</td>
<td>0.72 b</td>
<td>0.003 a</td>
</tr>
<tr>
<td></td>
<td>RSM 3</td>
<td>7.59 f</td>
<td>5.05 c</td>
<td>19.6 g</td>
<td>0.02 bc</td>
<td>0.05 bc</td>
<td>0.81 b</td>
<td>0.002 a</td>
</tr>
<tr>
<td>DSI-UP</td>
<td>Skim</td>
<td>56.5 d</td>
<td>0.35 e</td>
<td>103 d</td>
<td>0.01 bc</td>
<td>0.02 d</td>
<td>1.05 a</td>
<td>0.01 a</td>
</tr>
<tr>
<td></td>
<td>RSM 1</td>
<td>16.4 e</td>
<td>0.51 d</td>
<td>34.3 cd</td>
<td>0.005 c</td>
<td>0.10 a</td>
<td>1.20 a</td>
<td>0.01 a</td>
</tr>
<tr>
<td></td>
<td>RSM 2</td>
<td>70.3 c</td>
<td>0.37 e</td>
<td>183 c</td>
<td>0.01 bc</td>
<td>0.07 ab</td>
<td>1.11 a</td>
<td>0.01 a</td>
</tr>
<tr>
<td></td>
<td>RSM 3</td>
<td>143 a</td>
<td>0.37 e</td>
<td>383 a</td>
<td>0.01 bc</td>
<td>0.05 bc</td>
<td>1.06 a</td>
<td>0.01 a</td>
</tr>
<tr>
<td>IND-UP</td>
<td>Skim</td>
<td>47.0 d</td>
<td>19.7 a</td>
<td>81.7 e</td>
<td>0.01 bc</td>
<td>0.02 d</td>
<td>1.03 a</td>
<td>0.002 a</td>
</tr>
<tr>
<td></td>
<td>RSM 1</td>
<td>16.1 e</td>
<td>17.2 ab</td>
<td>38.4 f</td>
<td>0.01 bc</td>
<td>0.04 bc</td>
<td>0.92 ab</td>
<td>0.002 a</td>
</tr>
<tr>
<td></td>
<td>RSM 2</td>
<td>67.1 c</td>
<td>19.8 a</td>
<td>116 b</td>
<td>0.02 ab</td>
<td>0.04 bc</td>
<td>0.90 ab</td>
<td>0.003 a</td>
</tr>
<tr>
<td></td>
<td>RSM 3</td>
<td>123 b</td>
<td>14.0 b</td>
<td>310 b</td>
<td>0.02 bc</td>
<td>0.04 bc</td>
<td>0.85 ab</td>
<td>0.002 a</td>
</tr>
</tbody>
</table>

| P value   | Heat     | <.0001         | <.0001         | <.0001           | 0.049           | 0.013          | 0.001       | 0.157                   |
|           | Protein  | <.0001         | 0.771          | <.0001           | 0.124           | <.0001         | 0.277       | 0.110                   |
|           | Heat x Protein | <.0001    | <.0001         | <.0001           | 0.008           | <.0001         | 0.005       | 0.335                   |

1 Relative mean concentration triplicate (µg/kg).
2 HTST = high temperature short time; DSI-UP = ultrapasteurization by direct steam injection; IND-UP = ultrapasteurization by indirect heating; skim = milk with 0.1% fat; RSM = reformulated skim milk blended with micellar casein concentrate (MCC) and serum protein isolate (SPI).
3 Means in the same column followed by a different superscript indicate significance (P < 0.05).
Table 3.5. Best estimate threshold (BET) values for each odorant in skim milk

<table>
<thead>
<tr>
<th>Compound</th>
<th>BET(^2) (µg/L)</th>
<th>Previously reported threshold values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skim milk</td>
<td>Water</td>
</tr>
<tr>
<td>Hydrogen sulfide</td>
<td>22.5</td>
<td>10 µg/kg(^3)</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>35.2</td>
<td>5 µg/kg(^4)</td>
</tr>
</tbody>
</table>

1 Skim milk was HTST pasteurized at 77°C for 25 s.
2 Geometric means of group BET values from 38 panelists.
3 Pippen and Mecchi, 1969.
4 Vazquez-Landaverde et al., 2006.
6 EPA, 2016. The unit was converted from mg/m\(^3\).
Table 3.6. Descriptive sensory profiles of skim milk model system

<table>
<thead>
<tr>
<th>Samples</th>
<th>Overall aroma</th>
<th>Sweet aromatic</th>
<th>Cooked/milky</th>
<th>Sulfur/burnt</th>
<th>Eggy</th>
<th>Sweet taste</th>
<th>Astringent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk&quot;</td>
<td>2.0 d</td>
<td>1.2 a</td>
<td>3.2 b</td>
<td>ND(^4)</td>
<td>ND</td>
<td>2.3 a</td>
<td>2.0 a</td>
</tr>
<tr>
<td>Skim + CS(_2) added(^3)</td>
<td>3.0 c</td>
<td>0.9 b</td>
<td>3.7 a</td>
<td>1.1 a</td>
<td>ND</td>
<td>2.3 a</td>
<td>2.0 a</td>
</tr>
<tr>
<td>Skim + H(_2)S added(^3)</td>
<td>3.5 b</td>
<td>1.0 ab</td>
<td>3.5 a</td>
<td>ND</td>
<td>2.0 a</td>
<td>2.3 a</td>
<td>2.0 a</td>
</tr>
<tr>
<td>Skim + CS(_2) and H(_2)S added(^3)</td>
<td>4.3 a</td>
<td>0.5 c</td>
<td>3.7 a</td>
<td>0.8 a</td>
<td>1.9 a</td>
<td>2.3 a</td>
<td>2.0 a</td>
</tr>
</tbody>
</table>

1 Attribute intensities were scored on a 0 to 15-point universal intensity scale (Meilgaard et al., 2007). Fluid milk flavors fall between 0 and 4 on this scale (Drake, 2007; Croissant et al., 2007; McCarthy et al., 2017).
2 Skim milk was HTST pasteurized at 77°C for 25 s.
3 Carbon disulfide (CS\(_2\)) and/or hydrogen sulfide (H\(_2\)S) were added in skim milk at 50 µg/L and 30 µg/L, respectively.
4 ND = not detected.
5 Means in the same column followed by a different superscript indicate significance (\(P < 0.05\)).
Figure 3.1. Partial least squares correlation biplot (principal components 1 and 2) of volatile sulfur compounds and sensory attributes in skim milks and reformulated skim milks (RSM) at d1 post processing at 4°C. HTST = high temperature short time, DSI = ultrapasteurization by direct steam injection; IND = ultrapasteurization by indirect heating.
CHAPTER 4: FORMATION OF SULFUR FLAVOR COMPOUNDS IN ULTRAPASTEURIZED FLUID MILK FROM THE REACTION OF SULFUR CONTAINING AMINO ACIDS IN A SKIM MILK MODEL

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The content of this chapter will be submitted for publication in:

Journal of Dairy Science
Abstract

Volatile sulfur compounds (VSC) generated during ultrapasteurization (UP) of milk are responsible for eggy and sulfur/burnt off-flavors. A previous study demonstrated the source of sulfur compounds in milk during UP treatment and confirmed that hydrogen sulfide and carbon disulfide specifically contributed to eggy and sulfur/burnt flavors. However, the formation and precursors of VSC have not been addressed in milk processed by UP. The objective of this study was to elucidate the formation of VSC in UP milk via thermal reactions of lactose and lactose degradation products with cysteine or methionine in a skim milk model. Aqueous solutions of 0.01% cysteine and 0.09% methionine (w/w) both individually and as a blend, with or without 4.7% lactose (w/w) were first evaluated to clarify the role of lactose in VSC. Subsequently, a skim milk model (SMM) of fresh fluid milk permeate with added 0.01% cysteine, and or 0.09% methionine (w/w) was evaluated to confirm the presence of VSC in a model system, which closely mimicked skim milk. The origin and formation of VSC were then proposed using an equimolar solution of [1-^{13}C]-lactose/lactose, [^{13}C_6]-glucose/glucose, or [^{13}C_6]-galactose/galactose with cysteine or methionine, respectively. All of the mixtures were reacted using a bench top UP system. Targeted VSC in reactants were evaluated on the same day of reaction using solid phase microextraction (SPME) followed by gas chromatography-triple quadrupole mass spectrometry (GC-MS/MS) combined with a sulfur selective flame photometric detector. A comparison of the yield of VSC suggested that lactose played a significant role in the formation of VSC as methionine with lactose had the highest yield of methional, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide compared to mixtures containing methionine alone or blend of cysteine/methionine with or without lactose ($P < 0.05$). The yield of hydrogen sulfide and carbon disulfide were attributed to mixtures containing both cysteine and
cysteine/lactose. VSC yields from aqueous and SMM were consistent ($P > 0.05$). The $^{13}$C-label incorporation analyses showed that methional and dimethyl disulfide originated from [1-$^{13}$C]-lactose, whereas dimethyl sulfide and dimethyl trisulfide were found from unlabeled lactose, suggesting that their formation involves different routes from methional and dimethyl disulfide or methionine. Carbon disulfide was not found from labeled lactose/cysteine solutions revealing that its origin was from cysteine degradation alone along with the release of hydrogen sulfide.

Consistent patterns were observed from $[^{13}$C$_6]$-glucose/glucose or $[^{13}$C$_6]$-galactose/galactose with cysteine or methionine. These results propose precursors and formation of volatile sulfur compounds in UP fluid skim milk, and provide information on the role of methionine, cysteine and lactose in volatile sulfur flavor formation in UP milk.

**Key words:** fluid milk model system, volatile sulfur compounds, ultrapasteurization
Introduction

Volatile sulfur compounds (VSC) in milk significantly affect flavor and consumer acceptance of ultrapasteurized (UP) milk (Lee et al., 2017; Jo et al., 2018). A number of studies have characterized various sulfur compounds in UP milk, including hydrogen sulfide, carbon disulfide, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide (Vazquez-Landaverde et al., 2006; Al-Attabi et al., 2014; Jo et al., 2018). Jo et al. (2018, 2019) recently demonstrated that hydrogen sulfide and carbon disulfide were responsible for the distinct sulfur and eggy flavors in UP milk and confirmed serum protein as the primary source of these sulfur compounds. The increase of these compounds following the UP of milk can be attributed to the sulfur amino acid profile in serum protein, specifically cysteine and methionine (Belitz et al., 2009).

The contribution of cysteine and/or methionine to the sulfur flavors in various food matrices has been widely discussed with meat (Güntert et al., 1990; Werkhoff et al., 1990), eggs (Warren et al., 1995), fruits (Dirinck et al., 1981; Wyllie and Leach, 1992) and vegetables (Dateo et al., 1957; Maruyama, 1970). In UP milk, those amino acids are thought to be the key precursors present in serum protein generating volatile sulfur compounds (Belitz et al., 2009; Jo et al., 2018). It is also presumed that a probable route to the formation of sulfur compounds from these amino acids is associated with the Maillard reaction and thermal decomposition of precursors (Al-Attabi et al., 2009). However, no direct evidence has been provided for this pathway, especially in UP milk (> 138°C for at least 2 sec).

The main goal of flavor and flavor chemistry studies are the identification of aroma-active compounds that contribute to desirable and undesirable flavors and further characterization of their precursors and clarification of mechanisms controlling the formation of specific flavor compounds. Approaches using isotope labeling experiments have been introduced
to elucidate Maillard reaction pathways of flavor compounds (Schieberle, 2005; Cerny, 2008). The model mixture consists of potential precursors which are labeled or unlabeled amino acids and/or reducing sugars. The resulting labeled volatile compounds can identify the labeling position in the molecule and demonstrate the formation pathway (Cerny, 2008), which are quantitatively determined by an increase in the molecular mass of the target molecule based on mass spectrometry data (Schieberle, 2005). In the traditional approach, labeled precursors, mostly carbohydrates, were used to obtain information about the origin of the generated aroma compounds under Maillard-type reactions (Schieberle, 2005). More recently, a use of a mixture of unlabeled and fully labeled $^{13}$C carbohydrates has been developed to evaluate many possible different pathways to target compounds (Schieberle, 2005). The reaction systems using labeled compound mixtures have been used to propose the formation of flavor compounds including 5-(hydroxymethyl)-2-furaldehyde (HMF), phenylacetaldehyde, 2-methyl-3-furanthiol, pyrazines, and furans in application of heat processed foods and meat-like flavors (Locas et al., 2008; Amrani-Hemaili et al., 1995; Cerny, 2007; Wang and Ho, 2008; Wang et al., 2012; Hofmann et al., 2000).

A model mixture with all of the potential precursors at the same concentrations as in the target food matrix is a practical way to confirm key flavor compounds (Cerny, 2008). The use of model systems allows identification of previously unknown or postulated intermediates during reaction, especially the Maillard reaction (Cerny, 2008). The reaction of sulfur containing amino acids, cysteine or methionine, with sugars has been applied to generate meat-like flavors (Mottram and Nobrega, 2002; Cerny, 2007; Wang et al., 2012) and to elucidate the degradation of precursors and formation of corresponding flavor compounds (Yu and Ho, 1995; Zhang and Ho, 1991). However, these systems cannot be applicable to milk pasteurization since the time...
and temperature profile of UP milk have not been evaluated nor have these systems been evaluated with the sugar and mineral profile of milk. As such, the appropriate aqueous model system for milk can be developed to minimize the interference from buffers that may affect the rate of the reaction and the formation of volatile compounds. In order to demonstrate the relative contribution of lactose and sulfur amino acids to the formation of sulfur flavor compounds in milk, labeled standards can be evaluated in milk permeate to track the origin of the carbons in the volatile sulfur compounds. The objective of the current study was to elucidate the formation of volatile sulfur compounds responsible for generation of sulfur and eggy flavors in UP milk using skim milk model systems.

Materials and Methods

Materials

All chemical standards used in the study were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade water was purchased from Fisher Scientific (Hampton, NH). Skim milk permeate was obtained from a 2-stage ultrafiltration (UF) that was used to produce liquid serum protein isolate (SPI) described by Cheng et al. (2018). The composition of UF permeate (0.05% ± 0.004 fat, 4.76% ± 0.21 lactose, 0.13% ± 0.02 protein, and 5.74% ± 0.19 total solids) was measured using a Fourier-transform mid-infrared milk analyzer (LactoScope FTIR; Delta Instruments BC, Drachten, the Netherlands).

Experiment Overview

Two experiments were included in this study. Experiment 1 was conducted to clarify the role of lactose in VSC formation and the contribution of amino acid precursors to VSC in skim
milk model (SMM). The purpose of experiment 2 was to elucidate the formation pathway of target sulfur compounds.

**Experiment 1: VSC formation via thermal reactions of cysteine and methionine in aqueous and a skim milk model (SMM)**

**Model Reaction.** A model system was prepared by dissolving cysteine or methionine with and without lactose in aqueous solution or skim milk permeate obtained from UF (Table 4.1). The ratio of substrates was the ratio in milk (Belitz et al., 2009). Aqueous solutions of 0.01% L-cysteine and 0.09% L-methionine (w/w) were evaluated both individually and as a blend, with or without 4.7% lactose (w/w) in HPLC grade water to clarify the role of lactose in VSC formation. Then, SMM was evaluated and consisted of fresh fluid milk permeate with added 0.01% cysteine and/or 0.09% methionine (w/w). The purpose of using skim milk permeate as a base for SMM was to mimic skim milk with its mineral and lactose concentrations and to confirm the presence of sulfur compounds in heated skim milk.

All mixtures were adjusted to pH 6.7 by using 0.01N hydrochloric acid (HCl) (J. T. Baker, Phillipsburg, NJ) or 0.01N sodium hydroxide (NaOH) (Sigma-Aldrich) solutions. Five (5) mL of each mixture was transferred to a 20 mL amber vial with steel screw top containing silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA). A lab-scale bench top UP process of model mixtures was modified from previous studies (Morales et al., 1995; Kokkinidou and Peterson, 2013; Troise et al., 2014). The process was performed using two oil baths filled with silicone oil; one for the pre-heating (oil bath 1) and the other one for the final heating (oil bath 2). Time and temperature profile were monitored using blank solutions (water and UF permeate) with a calibrated thermocouple (Figure 4.1). Each vial was placed into oil bath
1 and preheated at 150°C for 30s then transferred to oil bath 2 and subsequently heated at 180°C for 60s. After heat treatment, each vial was immediately cooled to 10°C using liquid nitrogen and stored in a water bath filled with crushed ice to prevent further reactions. Instrumental volatile analysis was performed on the same day of reaction. The reaction experiments were replicated twice.

**HS-SPME-GC-MS/MS.** Selected sulfur compounds in aqueous or SMM including hydrogen sulfide and carbon disulfide, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, and methional (Jo et al., 2018) were measured using headspace solid-phase microextraction (HS-SPME) coupled with an Agilent 7890B gas chromatograph and an Agilent 7000C triple quad mass spectrophotometer (GC-MS/MS) and a sulfur selective flame photometric detector (FPD; Agilent Technologies Inc., Santa Clara, CA) equipped with a ZB-5ms column (30 m length x 0.25 mm i.d. x 0.25 µm film thickness; Phenomenex, Torrance, CA). Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (CTC Analytics, Zwingen, Switzerland). Twenty (20) µl of internal standard (ethyl methyl sulfide in ethyl ether at 1.65 mg/kg) was added to vials included five (5) ml of reactants. The analytical conditions and the multiple reaction monitoring (MRM) transition for selected sulfur compounds were followed as described by Jo et al. (2018). Dwell times were set to ensure 3-3.1 cycles over a peak.

MassHunter Qualitative and Quantitative Analysis software (Agilent Technologies Inc.) were used for data analysis. The relative concentration of each compound was calculated based on the response ratio of the quantifier ion to that of internal standard. The relative concentration was then used to determine the relative contribution of tested amino acids and lactose to the yield of target sulfur compounds.
Experiment 2: Isotope labeling studies and the formation of VSC

Model Reaction. A model system was prepared by dissolving cysteine or methionine with labeled or unlabeled lactose in aqueous solution using HPLC grade water (Fisher Scientific) (Table 4.2). An equimolar solution (1 mmol) of cysteine and methionine were mixed with the combination of labeled (0.5 mmol) and unlabeled lactose (0.5 mmol). Labeled D-[1-13C]-lactose (99% enrichment) labeled at C-1 and D-lactose (D-12C6-lactose) were prepared in a 1:1 ratio to understand the role of lactose and fragmentation of the sugar skeleton involved in the formation of VSC. Then equimolar solutions (1 mmol) of cysteine or methionine blended with the combination of D-[13C6]-glucose (99% enrichment; 0.5 mmol) and D-glucose (D-[12C6]-glucose; 0.5 mmol), or D-[13C6]-galactose (99% enrichment; 0.5 mmol) and D-galactose (D-[12C6]-galactose; 0.5 mmol) were prepared to demonstrate the contribution of lactose degradation products to VSC in milk. All mixtures were adjusted to pH 6.7 by using 0.01N HCl or 0.01N NaOH solutions. Five (5) mL of each mixture was transferred to a 20 mL amber vial with a steel screw top containing silicone septa faced in Teflon (Microliter Analytical). Lab-scale bench top UP processing of model mixtures was performed as previously described. Instrumental volatile analysis was performed on the same day of reaction. The experiment was performed in duplicate.

HS-SPME-GC-MS/MS. Selected sulfur compounds in reactants were measured using HS-SPME-GC-MS/MS as described in experiment 1. The product ions and MRM transition for target sulfur compounds were modified from experiment 1 based on the increase of mass unit from labeled compounds. Dwell times were set to ensure 3-3.1 cycles over a peak. MassHunter Qualitative and Quantitative Analysis software (Agilent Technologies Inc.) were used for data analysis. The proportion of isotopomer was calculated based on the abundance of product ion
signals of the respective target compounds and the values were corrected for $^{13}\text{C}$ natural abundance.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) with means separation. Differences between sample means were analyzed using Fisher’s least significant difference (LSD) test. All statistical analyses were performed using XLSTAT (version 2018, Addinsoft, New York, NY) at $P < 0.05$ significance.

Results and Discussion

The comparison of VSC from the reaction between cysteine and methionine with or without lactose

Targeted volatile sulfur compounds were generated using bench top UP to investigate the relative contribution of sulfur amino acids and lactose to their formation in UP milk. As shown in Figure 4.1, the time-temperature profile of the simulated UP was similar to that of previous studies using bench top UP (Kokkinidou and Peterson, 2013; Troise et al., 2014) as well as pilot plant scale UP by indirect heating (Lee et al., 2017).

Volatile sulfur compounds generated from the aqueous solutions containing cysteine and/or methionine with or without lactose were significantly different between amino acids (Figure 4.2; $P < 0.05$). Comparing the relative contribution of amino acids to sulfur compound formation in the aqueous system, methionine with lactose was more likely responsible for dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, and methional ($P < 0.05$). Methional was also produced from the degradation of methionine itself. However, the addition of lactose to
methionine significantly increased methional. Similarly, Yu and Ho (1995) reported that the formation of methional was favored in a methionine/glucose model system compared to methionine alone. Methional is known to be the important volatile compound that is the initial product of methionine degradation followed by further degradation into dimethyl disulfide and dimethyl sulfide (Ballance, 1961; Casey et al., 1965). Sulfur compounds including dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide were also produced at higher relative abundance from model systems containing methionine/lactose than methionine alone. The formation of hydrogen sulfide and carbon disulfide in aqueous model system were likely derived from models containing cysteine than methionine. Hydrogen sulfide was higher in model systems with both cysteine/lactose and cysteine alone compared to methionine and methionine/lactose (Figure 4.2), which indicates that hydrogen sulfide can be formed via multiple routes including cysteine degradation and Strecker degradation of cysteine with lactose (Mottram and Nobrega, 2002).

The application of SMM using milk permeate showed consistent sulfur compound profiles with aqueous solutions (Figure 4.3). Using SMM was a critical step to verify a consistent profile of sulfur compounds between aqueous and SMM, since aqueous models in this study were unbuffered and did not mimic a matrix close to skim milk. More importantly, the use of buffers in model systems can influence the rate of the reaction and produce more volatile compounds (Bobbio et al., 1973; Potman and van Wijk, 1989; Mottram and Nobrega, 2002). As such, reactions using milk permeate could be an alternative option for a buffered matrix for the objectives of this study. As consistent patterns were observed in VSC profile between aqueous solutions and SMM, it is confirmed that either unbuffered or buffered matrices can be used to evaluate the presence of VSC in UP milk model systems. In SMM, the reaction mixtures containing methionine had the highest yield of dimethyl sulfide, dimethyl disulfide, dimethyl
trisulfide, and methional compared to the mixtures containing cysteine or a blend of cysteine/methionine. This result further confirms that methionine is the primary source of those compounds in milk. Formation of hydrogen sulfide and carbon disulfide were prominent in the milk permeate with added cysteine followed by blends of cysteine and methionine, consistent with the aqueous model system. The primary precursor for hydrogen sulfide and carbon disulfide is likely to be cysteine, similar to results from aqueous solutions.

In both aqueous and SMM reactions, the formation of sulfur compounds in the UP milk model system mainly depended on the amino acids, cysteine and methionine, and lactose. More interestingly, it appeared that lactose played a critical role in the formation of sulfur compounds. The reaction between reducing sugars and amino groups of amino acids has been widely studied in various food matrices, however, the role of lactose in milk flavor has not been well studied. It has been suggested that lactose is the primary source for the Maillard reaction in milk (Hohno and Adachi, 1982; Kramhöller et al., 1993). However, studies on browning and the Maillard reaction showed that reaction products and intermediates obtained from disaccharides (e.g. lactose) were different from monosaccharides (e.g. glucose and galactose), which suggests that lactose may be one of the critical factors of milk volatile flavor compounds (Kato et al., 1988; Troyano et al., 1992; Hollnagel and Kroh, 2000; Brands and van Boekel, 2003; Hellwig et al., 2010). Studies on the Maillard reaction with lactose reported that lactose induced a slow and weak Maillard reaction compare to glucose or galactose because it was difficult to cleave the glycosidic bond, which can block the reaction and further determine the intermediates and reactivity throughout the Maillard reaction (Kato et al., 1988, 1989). As such, it would be expected that multiple pathways for flavor compound formation could exist in milk, particularly via Maillard reactions.
The reaction of isotope labeled precursors and the formation of VSC

GC-MS has been widely applied to studies using labeled precursors to compare the mass spectra and differences between compounds with labeled standards and unlabeled standards (Keyhani and Yaylayan, 1996; Cerny and Davidek, 2003; Cerny, 2007; Locas and Yaylayan, 2008). Especially, mass spectrometry is one of the primary tools to characterize isotope compositions (e.g. $^{12}\text{C}$ and $^{13}\text{C}$) of the labeled compound. In the spectra, the isotopic compositions of the labeled target compound are shown as isotopomers, which are an increase in the molecular mass or mass to charge ($m/z$) by one unit such as M, M+1, M+2, etc. (Schenk et al., 2016). Mass spectrometry determines the labeled position in the compound based on the measurement of isotope abundance and the calculation of the unlabeled to labeled isotope ratio.

Since the targeted compounds in the current study are sulfur compounds, which are low in mass, obtaining clear spectra without interference (e.g. chemical noise from matrix and other ions) is important. As such, utilizing MS/MS maximizes the identification of the product ions, including isotopomers, from the mixtures. This process further provides accurate intensity and quantification of product ions, which are required to calculate the isotope ratio.

The use of D-[1-$^{13}\text{C}$]-lactose in a model system estimated the formation of VSC based on the proportion of [$^{13}\text{C}$] atoms in product ions by GC-MS/MS (Table 4.3). The model system containing D-[1-$^{13}\text{C}$]-lactose with amino acids showed an increase in mass compared to the corresponding compounds produced from D-lactose (Table 4.3). Furthermore, D-[1-$^{13}\text{C}$]-lactose labeled at C-1 of glucose moiety would allow proposing which moiety in lactose (glucose or galactose) contributes to the formation of VSC and indicating the precursor of VSC in milk. For example, if VSC was generated from glucose only, incorporation of the $^{13}\text{C}$-label should be observed while no increased mass differences would be expected if it was generated from the
galactose moiety. The contribution of glucose or galactose as lactose degradation products to VSC formation was also confirmed using D-[\(^{13}\)C\(_6\)]-glucose and D-[\(^{13}\)C\(_6\)]-galactose. Comparison of the proportion of the isotopomers of VSC from the D-[\(^{1}\)\(^{13}\)C]-lactose and unlabeled lactose with methionine showed that methional was generated from methionine and the glucose moiety of lactose. Specifically, the incorporation of the [\(^{1}\)\(^{13}\)C] was observed in the product ion at \(m/z\) 77, which corresponds to the ion 76. The ratio between unlabeled and labeled methional was 49:51% at approximately 1:1 (Table 4.3), indicating that the glucose moiety in lactose was used as the carbon source for methional (Table 4.3). Similarly, the incorporation of [\(^{13}\)C] in dimethyl disulfide was also observed. The ratio of isotopomer between unlabeled and labeled dimethyl disulfide was 57:43% (Table 4.3), proposing that [\(^{13}\)C] from lactose remained as a carbon source for dimethyl disulfide. However, dimethyl sulfide and dimethyl trisulfide were not fully incorporated with [\(^{13}\)C] from labeled lactose, which implies that lactose is not a direct precursor for these compounds and it might be associated with different reaction routes such as further degradation or oxidation of methionine, methional or dimethyl disulfide. The reaction of D-[\(^{1}\)\(^{13}\)C\(_6\)]-lactose and unlabeled lactose with cysteine revealed that [\(^{13}\)C] was not incorporated with carbon disulfide formation, which suggests that lactose or glucose moiety of lactose may not be the primary carbon source in the reaction for this compound, consistent with results from cysteine alone and cysteine with unlabeled lactose in experiment 1 (Figures 4.2 and 4.3).

In order to obtain further insight of the effect of sugar fragmentation on the formation of VSC, D-[\(^{13}\)C\(_6\)]-glucose and D-glucose or D-[\(^{13}\)C\(_6\)]-galactose and D-galactose were reacted with cysteine or methionine, respectively (Tables 4.4 and 4.5). Similar to the previous result, the formation of methional was incorporated with [\(^{13}\)C] from labeled glucose, with the ratio of
which supports that the glucose moiety in lactose was the carbon source for methional. The D-\(^{13}\text{C}_6\)-galactose/D-glucose/methionine system also generated methional as the significant proportion of isotopomer was observed at the ratio of 38:62%. In contrast, dimethyl disulfide was exclusively unlabeled from the reaction of D-\(^{13}\text{C}_6\)-galactose/D-galactose/methionine system, indicating that galactose is not the precursor of the compound. However, in the D-\(^{13}\text{C}_6\)-glucose/D-glucose/methionine system, \(^{13}\text{C}\) was present at the ratio of 52:48% for dimethyl disulfide, which supports that glucose was the primary carbon source. For dimethyl sulfide and dimethyl trisulfide, the isotopomer proportion did not appear to be related to glucose or galactose as the carbon source. The reaction of cysteine with D-\(^{13}\text{C}_6\)-glucose/glucose or D-\(^{13}\text{C}_6\)-galactose/galactose revealed that neither glucose nor galactose were incorporated with the formation of carbon disulfide, again consistent with the previous experiments.

**The origin of VSC in milk**

The formation of methional from methionine and lactose is in agreement with previous studies from Yu and Ho (1995) and Ballance (1961). Although intermediates of lactose are known to be different from glucose and galactose (Kramhöller et al., 1993; Hellwig et al., 2010), methional was generated from lactose, glucose and galactose in the presence of methionine. Galactose reacted with methionine was not a primary precursor for dimethyl disulfide compared to methionine with lactose or glucose since labeled \(^{13}\text{C}\) was not observed in the compound. However, lactose and glucose contributed to the formation of dimethyl disulfide. This is possibly because of differences in sugar consumption rates during the reaction. Although it is known that galactose is a more reactive reducing sugar than glucose or lactose (Naranjo et al., 2013), Chen
et al. (2005) noted that glucose was consumed faster in the Maillard reaction while galactose was more involved with browning, which indicates that sugars may have different roles/kinetics in the Maillard reaction. This result would further suggest that lactose and lactose degradation products play a role in sulfur compound profiles in dairy products. Since lactose has the reactive carbonyl group on the glucose unit only, it would be involved in the formation of dimethyl disulfide.

As carbons from $^{13}\text{C}$ labeled lactose, glucose, and galactose were not integrated in dimethyl sulfide and dimethyl trisulfide, it would be possible to propose that these compounds are formed by further degradation of methional and dimethyl disulfide or methionine is the primary responsible precursor for these compounds in the presence of lactose. The precursor of dimethyl trisulfide has been suggested as methional (Gijs et al., 2000). Yu and Ho (1995) reported that the formation of dimethyl sulfide and dimethyl trisulfide were more favored by glucose/methionine sulfoxide, which is the oxidized form of methionine, compared to a glucose/methionine model system. Yu and Ho (1995) also noted that methionine sulfoxide can be formed by heat treatment or oxidizing agents. As such, it would be possible to assume that the formation of dimethyl sulfide and dimethyl trisulfide are likely involved with methionine oxidation during heat treatment or further degradation of methional and dimethyl disulfide, which also might be associated with their oxidation. In relation to UP treatment, milk processed by indirect heating method would be expected to be more favorable in the formation of these compounds, compared to direct steam injection, since it contains higher dissolved oxygen and can lead to further oxidation (Datta et al., 2002; Jo et al., 2018). Dimethyl sulfide was present at higher concentration in milk processed by UP-indirect heating than UP-direct steam injection (Al-Attabi et al., 2009; Jo et al., 2018). As such, it is possible that lactose, glucose or galactose
may not be direct precursors for dimethyl sulfide, but the presence of these sugars would be favored to generate more direct precursors of dimethyl sulfide including methional, dimethyl disulfide or methionine sulfoxide.

Since $[^{13}\text{C}]$ from lactose, glucose or galactose was not incorporated with carbon disulfide, cysteine would be the likely responsible precursor of carbon disulfide. However, even if lactose or other sugars are not directly integrated in carbon disulfide, they can be still important precursors because the cysteine/lactose model system generated higher amounts of carbon disulfide as well as hydrogen sulfide in the reactions of aqueous solutions and SMM. The presence of lactose or other sugars in milk could promote the formation of both hydrogen sulfide and carbon disulfide without being directly involved, as cysteine can undergo competitive reactions between Strecker degradation and subsequent degradation (e.g. decarboxylation) (Pripis-Nicolau et al., 2000).

**Conclusion**

Model systems containing cysteine or methionine with or without lactose in aqueous solution and skim milk model from milk permeate allowed clarification of the relative contribution of precursors to sulfur compounds in milk using bench top UP treatment. Both amino acids and lactose had significant effects on the formation of VSC; methionine/lactose led to the formation of methional, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide and cysteine and cysteine/lactose yielded higher amounts of hydrogen sulfide and carbon disulfide. The use of $[^{13}\text{C}]$ labeled lactose, glucose, and galactose in mixtures with cysteine or methionine showed that methional and dimethyl disulfide originated from methionine/lactose, whereas the formation of dimethyl sulfide and dimethyl trisulfide were involved with subsequent reactions
including oxidation. Although lactose is not a direct precursor of hydrogen sulfide and carbon disulfide, results suggest that lactose may promote the degradation or reaction of cysteine during heat treatment. These findings can provide information on the precursors and the formation of sulfur compounds in milk in relation to UP treatment.

Acknowledgements

This study was supported in part by the National Dairy Council (Rosemont, IL). The use of tradenames does not imply endorsement or lack of endorsement by those not mentioned.
REFERENCES


Table 4.1. Model reactions in experiment 1

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Aqueous</th>
<th>Skim milk model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-A</td>
<td>1-B</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-methionine</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>D-lactose</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

1 All mixtures were adjusted to pH 6.7 using 0.01N HCl or 0.01N NaOH.
2 All mixtures contained 0.01% (w/w) cysteine and/or 0.09% (w/w) methionine with or without 4.7% (w/w) lactose.
2 Skim milk model was prepared using milk permeate obtained from ultrafiltration (UF).
Table 4.2. Model reactions in experiment 2

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Amount (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-A</td>
</tr>
<tr>
<td>D-lactose</td>
<td>0.5</td>
</tr>
<tr>
<td>D-[1-\textsuperscript{13}C]-lactose</td>
<td>0.5</td>
</tr>
<tr>
<td>D-glucose</td>
<td></td>
</tr>
<tr>
<td>D-[\textsuperscript{13}C\textsubscript{6}]-glucose</td>
<td></td>
</tr>
<tr>
<td>D-galactose</td>
<td></td>
</tr>
<tr>
<td>D-[\textsuperscript{13}C\textsubscript{6}]-galactose</td>
<td></td>
</tr>
<tr>
<td>L-cysteine</td>
<td>1.0</td>
</tr>
<tr>
<td>L-methionine</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} All aqueous mixtures were adjusted to pH 6.7 using 0.01N HCl or 0.01N NaOH.
Table 4.3. Proportion of isotopomers in volatile sulfur compounds formed from the reaction of D-[1-\textsuperscript{13}C]-lactose and D-lactose with cysteine or methionine\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion (m/z)</th>
<th>Analyzed product ions (m/z)</th>
<th>Unlabeled carbon atoms (%)</th>
<th>Labeled carbon atoms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-\textsuperscript{13}C]-D-lactose/L-cysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>76</td>
<td>46; 47</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>[1-\textsuperscript{13}C]-D-lactose/L-methionine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
<td>62</td>
<td>47; 48</td>
<td>73</td>
<td>27</td>
</tr>
<tr>
<td>Dimethyl disulfide</td>
<td>94</td>
<td>79; 80</td>
<td>57</td>
<td>43</td>
</tr>
<tr>
<td>Dimethyl trisulfide</td>
<td>126</td>
<td>80; 81</td>
<td>82</td>
<td>18</td>
</tr>
<tr>
<td>Methional</td>
<td>104</td>
<td>76; 77</td>
<td>49</td>
<td>51</td>
</tr>
</tbody>
</table>

\textsuperscript{1} D-[1-\textsuperscript{13}C]-lactose was labeled at C-1 of glucose moiety.

\textsuperscript{2} The proportion of isotopomer was calculated based on the abundance of analyzed product ion signals and corrected for \textsuperscript{13}C natural abundance.
Table 4.4. Proportion of isotopomers in volatile sulfur compounds formed from the reaction of D-[\textsuperscript{13}C\textsubscript{6}] glucose and D-glucose with cysteine or methionine\textsuperscript{1}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion ((m/z))</th>
<th>Analyzed product ions ((m/z))</th>
<th>Unlabeled carbon atoms (%)</th>
<th>Labeled carbon atoms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-[\textsuperscript{13}C\textsubscript{6}] glucose /D-glucose/L-cysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>76</td>
<td>46; 47</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>D-[\textsuperscript{13}C\textsubscript{6}] glucose /D-glucose/L-methionine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
<td>62</td>
<td>47; 48</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>Dimethyl disulfide</td>
<td>94</td>
<td>79; 80</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>Dimethyl trisulfide</td>
<td>126</td>
<td>80; 81</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Methional</td>
<td>104</td>
<td>76; 77</td>
<td>51</td>
<td>49</td>
</tr>
</tbody>
</table>

\textsuperscript{1} The proportion of isotopomer was calculated based on the abundance of analyzed product ion signals and corrected for \textsuperscript{13}C natural abundance.
Table 4.5. Proportion of isotopomers in volatile sulfur compounds formed from the reaction of D-[^13]C₆-galactose and D-galactose with cysteine or methionine¹

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion (m/z)</th>
<th>Analyzed product ions (m/z)</th>
<th>Unlabeled carbon atoms (%)</th>
<th>Labeled carbon atoms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>D-[^13]C₆]-galactose/D-galactose/L-cysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>76</td>
<td>46; 47</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>D-[^13]C₆]-galactose/D-galactose/L-methionine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
<td>62</td>
<td>47; 48</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>Dimethyl disulfide</td>
<td>94</td>
<td>79; 80</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Dimethyl trisulfide</td>
<td>126</td>
<td>80; 81</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>Methional</td>
<td>104</td>
<td>76; 77</td>
<td>38</td>
<td>62</td>
</tr>
</tbody>
</table>

¹ The proportion of isotopomer was calculated based on the abundance of analyzed product ion signals and corrected for ^13C natural abundance.
Figure 4.1. Time-temperature profile of aqueous and skim milk models (SMM) reactions using lab-scale bench top ultrapasteurization (UP). Bench top UP was modified from Morales et al., 1995; Kokkinidou and Peterson, 2013; Troise et al., 2014.
Figure 4.2. Relative concentration of volatile sulfur compounds from the aqueous model reactions in experiment 1. Cys = L-cysteine; Lac = D-lactose; Met = L-methionine. Means not sharing common letters are different ($P < 0.05$). Error bars represent standard error.
Figure 4.3. Relative concentration of volatile sulfur compounds from the skim milk model (SMM) reactions in experiment 1. Cys = L-cysteine; Lac = D-lactose; Met = L-methionine. Means not sharing common letters are different ($P < 0.05$). Error bars represent standard error.