2012

Effects of thermosonication on proteases and characteristics of milk and cream

Sakthi Vijayakumar
Iowa State University

Effects of thermosonication on proteases and characteristics of milk and cream

by

Sakthi Vijayakumar

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Stephanie Clark, Major Professor
Stephanie Jung
David Grewell

Iowa State University

Ames, Iowa

2012

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my academic advisor and supervisor, Dr. Stephanie Clark, for her continuous support, patience, enthusiasm and motivation all through my graduate research and academic work. I would have been lost without her teaching, ideas, and encouragement.

I would also like to thank my committee members, Dr. David Grewell and Dr. Stephanie Jung, for their technical guidance and support on my research experiments. Their insightful comments helped me in all the time of research and writing of this thesis.

I am grateful to Dr. Pamela White, Dr. Lester Wilson, and Dr. Wendy White for sharing their labs with me for my experiments and to Dr. Patricia Murphy for helping me troubleshoot the problems I encountered in enzyme assay.

My sincere thanks also go to Dr. Hui Wang, CCUR Pilot Plant Manager, for helping me run the cream separator in the Pilot Plant, Devin Maurer, for helping me with rheological experiments and particle size analysis, Melissa Montalbo-Lomboy and Priyanka Chand for their support with setting up ultrasonication experiments, Lumeng Jin, Xiaoxiao Wang, Gerui Li, Babu Chinnasamy, Katherine Lynch and Brittney Fritz for their help with microbial analysis, Siqi Wang for her help with color measurements and Linda Berlakovich, Eric Testroet and Siyu Qian for their assistance in sensory evaluation. I would also like express my thanks to the QDA panelists for their continuous participation in the sensory evaluation study.

Last but not the least, I would like to thank my parents, brother, friends and husband for supporting me throughout my life and helping me through difficult times. To them I dedicate this thesis.

ABSTRACT

Shelf-life of pasteurized milk is limited by heat-stable proteases, which cause gelation and bitterness. Ultra-high temperature processing inactivates proteases, but detrimentally affects milk quality. An adjunct to pasteurization is sought to extend milk shelf-life, while maintaining sensory properties. In this study, we evaluated the effects of combined heat and ultrasound on the activity of *Staphylococcus aureus* protease and total plasmin, as well as the impact on sensory properties of milk and cream. Sonication at 133 µm (p.p) for 2.5 min decreased the enzyme activity by approximately 72% in pasteurized skim and whole milk and by 92% in commercial pasteurized reduced-fat milk. Viscosity of commercial pasteurized milk samples that were thermosonicated at 133 µm (p.p) for 2.5 min was not affected. However, off-aromas (cooked and plastic/rubbery) were observed in commercial pasteurized reduced-fat and whole milk treated with 133 µm (p.p) for 2.5 min. Thermosonication at 152 µm (p.p) for 3 min decreased the plasmin activity by about 81 to 94% in raw skim milk and by 96% in raw cream. Enzyme activity in most raw skim milk samples measured at the end of 4 weeks was one- to two-fold higher than that on day 0, whereas in most raw cream samples enzyme decreased with storage time. Descriptive analysis of odor attributes of raw skim milk and raw cream that were thermosonicated at 152 µm (p.p) for 3 min was conducted for up to 4 weeks with 8 trained panelists. No significant differences were observed between the intensities of offensive eggy and rubbery odor attributes of thermsonicated skim milk and pasteurized commercial skim milk and cream ($p<0.05$). Lightness (L*) values of raw skim milk and cream were not adversely affected by thermosonication. Thermosonication did not adversely affect the viscosity of skim milk and cream samples when measured over the 4-week storage period. Thermosonication decreased the fat globule size in both skim milk and cream and the homogenizing effect increased with increase in treatment time. Thermosonication at 133 and 152 µm (p.p) for 1 and 3 min completely destroyed coliforms and destroyed over 99% of the total aerobic bacteria. The total aerobic bacteria count of thermosonicated skim milk and cream samples were less than 20,000 CFU/mL on day 30. Because thermosonication did not induce off-aromas or viscosity changes, but inactivated microorganisms and protease enzymes, thermosonication may be an appropriate adjunct to pasteurization to extend milk shelf-life.

CHAPTER 1. INTRODUCTION

Milk is a wholesome food that is consumed widely in every part of the world. Health benefits gained from drinking fluid milk, especially in young children, have been emphasized since historical times. Fluid milk is a source of nine essential nutrients such as vitamins A, D, B12, riboflavin, niacin, calcium, potassium, phosphorus and protein. Because of the increasing demands for dairy products, the annual production of fluid milk in the U.S. has seen a 17% increase over the past ten years (USDA, 2011). Approximately one-third of the milk produced is being processed into fluid milk and cream and the remaining goes into making of other products such as cheese (USDA, 2009). While per capita consumption of whole fluid milk has declined over the years, consumption of skim milk, reduced fat milk and other dairy products such as cream, yogurt, cheese etc. have seen steady increases (ERS, 2003).

1.1. Shelf-life of Fluid Milk

Somewhat limiting its marketability, fluid milk is a highly perishable product, which competes with other beverages such as fruit juice, soda or other sugary drinks in the market that have a higher shelf-life. Thus milk processors and dairy scientists are keen on finding ways to improve the shelf-life of fluid milk. The definition of shelf-life has seen several revisions over time. The Institute of Food Science and Technology guidelines (IFST, 1993) defines shelf-life as the time period during which the food product, when stored at the recommended conditions, would remain safe, retain desired sensory, physical, chemical and microbiological characteristics, and conform to the nutritional data declared on the label.

Raw milk is a highly perishable product, with a shelf-life of approximately three to five days when stored under refrigerated conditions i.e. at 4°C. The shelf-life of raw milk depends on various factors, such as milk collection and handling techniques, cleanliness of milking environment, storage temperature, and somatic cell count and microbial load of milk. The shelf-life of processed fluid milk depends on various factors, such as quality of incoming raw milk, processing time and temperature, storage temperature, extent of exposure to light and post-processing contamination (Simon and Hansen, 2001; Tetra Pak, 2000). One or more of the above factors cause the presence and/or growth of spoilage microorganisms and spoilage

enzymes, which limit the shelf-life of processed milk. For safety and extended shelf-life, milk needs to be thermally pasteurized and stored under appropriate conditions. The extent of thermal processing determines the shelf-life of the milk. Conventional systems use High Temperature Short Time (HTST) continuous pasteurization to process fluid milk by heating it to at least 72°C and holding thereat for 15s. Alternatively, Low Temperature Long Time (LTLT) batch pasteurization is also followed, by heating the milk to at least 63°C and holding thereat for 30 min. Ultra high temperature processed milk is processed by taking the milk to up to 138°C and holding thereat for at least 2 s (FDA, 2009). High temperature short time or low temperature less time pasteurization destroys all the pathogens and many spoilage microorganisms and thus renders a shelf-life of approximately 10 to 21 days when bottled and stored under refrigerated conditions (Allen and Joseph, 1985; Chapman et al., 2001). Ultra high temperature processing can extend milk's shelf-life to at least 60 days at room temperature, however such extreme thermal treatments can affect color, flavor and nutritional properties of the milk. It is important that when investigating the potential of a processing technique in extension of shelf-life, processors should not compromise the sensory quality and nutritional properties of milk.

Manufacturers are in pursuit of raising margins through extended geographical coverage of product distribution. Milk is a perishable product and thus costs involved in refrigeration during transport and storage limits its distribution. The need for extended shelf-life milk has given rise to new processing techniques such as high pressure processing, ultrasonication, pulsed electric field, microwave, irradiation, etc.

When milk is pasteurized, all pathogenic microorganisms and most spoilage microorganisms and some native enzymes are destroyed. However, spores of thermoduric microorganisms such as psychrotrophs, and native and microbial enzymes such as proteases survive the heat treatment and cause spoilage of milk under extended storage. Today's improved post-processing product handling techniques have reduced contamination by gram negative psychrotrophs, which can limit the shelf-life of commercially pasteurized fluid milk to approximately 14 to 17 days. However, the presence of thermoduric gram-positive

microorganisms, such as *Bacillus sp.* and *Microbacter sp.* continue to limit the shelf-life of fluid milk (Fromm and Boor, 2004; Ralyea et al., 1998).

1.2. Indigenous Enzymes in Milk

Native milk enzymes enter milk from four major sources: blood plasma, secretory cell cytoplasm, milk fat globule membrane, and somatic cells (leucocytes) from the blood (Fox et al., 2003). Most enzymes in milk are associated with the milk fat globule membrane. Approximately 70 indigenous enzymes have been reported in bovine milk (Fox et al., 2003). Many enzymes in milk are inactive because of the absence of substrates, and/or presence of inhibitors or other unsuitable environmental conditions. Most of the indigenous enzymes do not possess any known role in synthesis and secretion of milk, and a few enzymes have a biologically important role only after secretion of milk. Lipoprotein lipase, protease, acid phosphatase and xanthine oxidase cause milk quality deterioration, while enzymes such as lactoperoxidase, sulphydryl oxidase, superoxide dismutase help to protect the quality. Alkaline phosphatase, which is inactivated at conventional pasteurization temperature, serves as an indicator for proper pasteurization. Enzymes such as catalase and N-acetyl-β-D-glucosaminidase can indicate the inflammation of mammary tissue i.e. presence of mastitis in cattle, and lysozyme possesses antimicrobial activity (Fox et al., 2003). In addition of indigenous enzymes in milk, enzymes are also produced by the microorganisms like *Pseudomonas* spp., *Bacillus* spp. etc. (Sorhaug and Stepaniak, 1997). These microorganisms enter the milk from the udder of cow, milking and milk handling practices and environment. Processing, packing and storage mainly influence the survival of these microorganisms. These microorganisms produce enzymes such as proteases, lipases etc. which are heat resistant and cause spoilage of milk. Because most indigenous milk enzymes and microbial enzymes are deleterious to milk quality and have no beneficial effect on the nutritional or sensory attributes of milk, inactivating these enzymes is one of the objectives of thermal processing.

1.3. Shelf-life Limiting Proteases

Proteases are one of the major types of enzymes that limit the shelf-life of fluid milk, apart from lipoprotein lipase, acid phosphatase and xanthine oxido-reductase (Fox and Kelly, 2006). Proteases hydrolyze milk proteins into various peptides and one result of these cleavage reactions is age gelation. The peptides are also responsible for bitterness, which often accompanies flocculation of aged milk. Proteases can be indigenous (the plasmin system) or produced by microorganisms that survive milk pasteurization or are post-pasteurization contaminants.

1.3.1. The Plasmin System

Plasmin (EC 3.4.21.7) is a fibrinolysin that is secreted into the milk from blood plasma through the mammary cells (Fox and Kelly, 2006; Kelly et al., 2006). Plasmin is the major indigenous proteinase found in milk apart from cathepsins and elastase (Bastian and Brown, 1996; Fox and Kelly, 2006; Kaminogawa and Yamauchi, 1972; Kelly et al., 2006; Pereda et al., 2008). The plasmin system consists of plasmin, plasminogen, plasminogen activators and inhibitors of plasminogen activators and of plasmin. The inhibitors are soluble in the milk serum while the rest of the plasmin system, i.e. plasminogen, plasmin and plasminogen activators, are associated with the casein micelles (Bastian and Brown, 1996; Fox and Kelly, 2006). Upon storage, plasminogen in milk is cleaved at the Arg^{557}-Ile^{558} peptide bond by proteases such as urokinase and tissue-type plasminogen activators (Bastian and Brown, 1996; Fox and Kelly, 2006). This proteolytic cleavage converts inactive plasminogen into active plasmin, and hence a higher plasmin activity is observed in pasteurized milk upon storage (Kelly et al., 2006). Further, the inhibitors are inactivated by commercial thermal pasteurization, while the activators are heat stable and survive pasteurization. Plasmin is optimally active at pH 6 and temperature of 37.7°C (Fox and Kelly, 2006; Kelly et al., 2006). Bovine milk pH ranges between 6.5 and 6.8 (Tetra Pak, 2000) making it ideal for plasmin activity. Storing pasteurized milk under refrigerated conditions helps to slow down the activity of plasmin, which is induced into action by pasteurization because inhibitors are inactivated by pasteurization and activators remain active).

1.4. Inactivation of Proteases

Plasmin is heat stable and survives pasteurization; UHT processing can only reduce plasmin activity but cannot completely inactivate the enzyme (Newstead et al., 2006). A minimum of 30% of the activity of plasmin exists in milk after UHT processing (Alichanidis et al., 1986). Heating to 120°C for 15 min can completely inactivate plasmin in milk (Chen et al., 2003) and heating to 85°C for 5 min can completely inactivate plasmin in phosphate buffer of pH 6.6 (Borda et al., 2004). This implies that plasmin inactivation depends on the media the enzyme is present in. Similar to plasmin, proteases produced by the psychrotophic and thermoduric bacteria that survive pasteurization are heat stable. Proteases produced by *Pseudomonas sp.* are stable to pasteurization and UHT processing (Malik and Swanson, 1974). Heat stable native and microbial proteases greatly limit the shelf-life of pasteurized milk and ways to inactivate these enzymes are being extensively investigated by scientists. Emerging technologies such as high pressure processing (Bilbao-Sainz et al., 2009; Borda et al., 2004; García-Risco et al., 2003), pulsed electric field (Bendicho et al., 2005), ultrasound (Vercet et al., 2002) etc. are being explored to investigate their potential to inactivate the shelf-life limiting enzymes in milk, particularly proteases.

1.5. Ultrasound in Food Processing

Ultrasound is a form of energy generated by cyclic sound pressure waves of frequencies that are greater than the upper limit of human hearing range, typically above 20 kHz (Patist and Bates, 2008). The application of ultrasound waves is called sonication. Ultrasound waves are commonly generated using transducers containing piezoelectric materials, such as barium titanate or lead matabionate, etc. When an electric field is applied across an array of piezoelectric crystals, mechanical displacement in the form of vibration is produced, resulting in generation of ultrasound.

1.5.1. Working Principle

When ultrasound waves are passed through a liquid substance or a biological substance such as food material, alternating regions of high and low pressure i.e. compression and

expansion, respectively, are created, which induce cavitation and form gas bubbles. These gas bubbles expand because of increased gas diffusion during the expansion cycle and rapidly condense (implosion) at one point when the energy of the ultrasound waves is insufficient to retain the vapor phase in the gas bubbles. The condensed molecules collide violently, resulting in shock waves (mechanical or shear forces), regions of high temperature and pressure, and generate free radicals through water sonolysis. In the fluid that is subject to sonication, cavitation exerts rotational forces and stresses on the cells in the vicinity of the gas bubbles. These result in a microscopic fluid movement called microstreaming. Cavitation is thus able to increase heat and mass transfer (Jayasooriya et al., 2004; Zheng and Sun, 2006). Destruction of microorganisms or inactivation of enzymes is caused by either one or combined action of the above-said consequences of sonication. However, the cavitation created depends on many factors, such as frequency and intensity of ultrasound waves, ambient temperature and pressure, product properties such as viscosity, surface tension etc.

Typically there are two types of ultrasound used in the food industry – high power and low power ultrasound. In the food industry, low power ultrasound applications involve frequencies in the range of 2-10 MHz and low power (up to 10 MHz). Low power ultrasound does not cause any physical or chemical alteration in the biological substance they pass through and is thus used for rapid, in-line quality monitoring by measuring physiochemical properties such as composition, structure and physical state of foods (Fellows, 2000; Jayasooriya et al., 2004; Knorr et al., 2004; McClements, 1995; Simal et al., 2003). High power ultrasound applications used in the food industry utilize frequencies in the range of 20 to 100 kHz (Jayasooriya et al., 2004; McClements, 1995). These waves can cause physical and chemical changes in the material they pass through and thus are typically used for emulsion generation, cell disruption and recently research to investigate their potential in enzyme inactivation, microbial destruction, modification of crystallization, enhancement of filtration and drying processes, etc. (Knorr et al., 2004; McClements, 1995; Roberts, 1993; Zheng and Sun, 2006).

1.5.2. Applications of Ultrasound in the Dairy Industry and Enzyme Inactivation

Ultrasound has a broad range of applications from quality control and enhancement to shelf-life extension in the dairy industry. Ultrasound can be used in the dairy industry for cleaning purposes (Kivelä, 1996). Ultrasound promotes nucleation and reduction of ice crystal size in ice cream (Zheng and Sun, 2006). Low power ultrasound can be used to measure the extent of crystallization and melting in emulsions (Povey and Mason, 1998). Ultrasound can also effectively reduce the size of fat globules (Martinez et al., 1987; Villamiel and de Jong, 2000) and is thus capable of producing stable emulsions. Wu et al. (2001) reported that ultrasound homogenizes milk better than the conventional system. One of the reasons for the homogenizing effects of ultrasound is the formation of eddy currents in the fluid that is being sonicated (Earnshaw et al., 1995; Floros and Liang, 1994). Homogenizing milk using ultrasound could increase the protein binding sites on the milk fat globule membrane and this in turn could improve cheese yield (Müller, 1992). Ultrasound treatment of milk prior to inoculation of yogurt cultures increased the water holding capacity and viscosity of yogurt and reduced the syneresis (Wu et al., 2001). The researchers also showed that ultrasound treatment after inoculation of yogurt cultures decreased the fermentation time by 0.5 h. High power ultrasound has proven to be useful in inactivating microorganisms (García et al., 1989; Ordóñez et al., 1987; Wrigley and Lorca, 1992;) and thus has a good potential in fluid milk pasteurization.

Ultrasound has been found to be useful in enzyme inactivation only when combined with other factors such as heat and/or pressure (Villamiel and de Jong, 2000). Protease produced by *P. flourescens* was resistant to manothermosonication (ultrasound combined with heat and pressure) at 30°C and was very sensitive at 76°C (Vercet et al., 2002). Researchers have also reported that ultrasonication (117 µm) at room temperature and pressure had no effect on lysozyme activity while applying 200 kPa pressure and increasing temperature between 60 and 80°C increased the inactivation rate (Manas et al., 2006) whereas a ten-fold decrease in lysozyme activity was observed when lysozyme was thermosonicated at 117 µm, 200 kPa and 70°C for 3.5 min. Researchers have observed that the D-value of pectinmethylesterase activity was 25.3 min during thermal inactivation whereas it was 0.3 min during

thermosonication at 72°C (57). They have also reported that thermosonication increased enzyme inactivation by 39- to 374-fold at 61°C and by 36- to 84-fold at 72°C when compared to thermal inactivation. A synergistic effect is observed when heat is combined with ultrasound and thus opens the door for many applications in the food industry, particularly in enzyme inactivation. Our major objective is to study the combined effect of heat and ultrasound on the activity of shelf-life limiting proteases in milk.

1.6. Sensory Evaluation

One of the important aspects of food processing, apart from ensuring food safety and improving shelf-life, is to improve or maintain the sensory characteristics of the product. Off-odors and flavors in milk can come from a variety of sources, such as cattle, feed, environment where milking is performed, post-pasteurization contamination, extent of exposure to light and oxygen, processing conditions, storage temperatures, packaging materials, etc. Unpleasant aromas and flavors in fluid milk can be an indication of milk spoilage (Hayes et al., 2002) and thus are important in determining the shelf-life of products. Often times, foods that are processed at extreme temperatures to make them shelf-stable have lower consumer acceptability when the sensory quality of the product is inferior. In the U.S., ultra-high temperature (UHT) milk has lower consumer acceptability when compared to pasteurized milk, in spite of the fact that it has extended shelf-life when compared to pasteurized milk. UHT milk is processed by heating it to approximately 138°C for at least 2 s (FDA, 2009); this extreme thermal processing condition not only destroys pathogens, spoilage microorganisms and inactivates proteases to some extent, but also deteriorates the quality of the milk by causing browning reactions and affects the sensory properties. Through UHT pasteurization, up to 60-d shelf-life can be achieved (Boor and Nakimbugwe, 1998), however, processing at high temperatures imparts strong cooked flavor and odor in the milk and can lead to Maillard or caramelization reactions (Clare et al., 2005). Consumer studies have shown that children of age 6 to 11 years found 2% UHT milk to be undesirable (Chapman et al., 2001). Heating milk results in an increase in sulfur compounds, thus leading to off-flavors (Christensen and Reineccius, 1992). Data on effects of ultrasound treatment on the sensory properties of milk are limited.

Studying the effects of ultrasound processing on the sensory properties of milk is of critical importance to understand ultrasound's prospective application in the dairy industry. Very few published works are found on the effects of ultrasound on sensory properties of foods. Hexanal and limonene are the key compounds that cause off-flavor in sunflower oil subjected to sonication at 150 W power (Chemat et al., 2004). Researchers reported that thermosonicating pasteurized homogenized milk of 1.5% fat content continuously at 24 kHz frequency, 400 W power and 45°C from 2.5 to 20 min resulted in an undesirable rubbery aroma (Riener et al., 2009). The concentration of volatile compounds such as 1-hexene, 1-octene, 1-nonene, 5-methyl-1,3-cyclopenatadiene, benzene, toluene, p-xylene, n-hexanal, and n-heptanal increased over the first 5 min of sonication and Gas Chromatography – Olfactometry evaluation showed that none of the compounds individually contributed to the rubbery aroma. The researchers further reported that the formation of short-chain alkenes could be because of pyrolysis of fatty acids and that sonication-induced lipid oxidation is a likely pathway for the formation of aldehydes, because hexanal and heptanal are compounds of free-radical-induced lipid oxidation. Riener et al. (2001) also reported that reducing the sonication power from 400 to 100 W resulted in a decrease in the intensity of rubbery aroma. It should be noted that Riener et al. (2001) reported the effects of thermosonication on a commercial sample that had been already pasteurized. Because the milk had undergone two steps of thermal processing, i.e. commercial pasteurization and thermosonication, formation of volatile compounds that create off-odors might be favored. Evaluating the effects of thermosonication on fresh raw milk's sensory properties can shed more light on the effects of thermosonication on the sensory properties of milk.

Quantitative descriptive analysis (QDA) is an approach to characterize sensory attributes; it has been widely used for dairy products such as pasteurized milk (Phillips et al., 1995; Quiñones et al., 1998), ultra-pasteurized milk (Chapman et al., 2001), ice-cream (Ohmes et al., 1998; Roland et al., 1999) and cheese (Ordóñez et al., 1998). Researchers have compared the sensory attributes of UHT milk and microwave processed milk using the QDA approach and reported that on a 5-point scale microwave processed milk products received a lower score on caramelized flavor, astringency and presence of brownish hues when compared to UHT milk (Clare et al., 2005). The principle of QDA is to broadly describe the specific

sensory attributes of a product on a quantitative basis using trained panelists (Meilgaard et al., 1999). Panelists are usually trained until their abilities in identifying different intensities of sensory attributes are reproducible and reliable; the QDA approach to product description relies heavily on statistical analysis. Use of QDA to provide an odor profile of thermosonicated skim milk and cream can help investigators to understand whether thermosonication induces offensive odors in milk and cream and whether the odors have an association with fat content. Thus one of our objectives is to investigate the effects of thermosonication on the sensory properties, odor profile in particular, of skim milk and cream using trained panelists.

1.7. Rheological Measurements

Rheological properties such as viscosity are important in fluid milk processing because they not only describe the fluid flow in processing but also play an important role in shelf-life of milk. Factors such as particle size (Floury et al., 2000), casein micelle aggregates (Walstra and Jenness, 1985) and heat denaturation of whey protein (Clare et al., 2005) can affect the viscosity of milk. Denatured whey proteins often undergo sulfhydryl-disulfide interchange reactions, which can increase milk viscosity. Changes in viscosity can greatly affect consumer acceptability. Few published works demonstrate the effects of processing on the viscosity of fluid milk. Application of pulsed electric field in the range of 45 to 55 $kVcm^{-1}$ decreased the viscosity of skim milk (Floury et al., 2006) and ultra high pressure homogenization (200 MPa) decreased the viscosity of whole milk (Pereda et al., 2007). No practical significant differences in viscosity values were found between UHT milk and microwave-treated milk (Clare et al., 2005). The effects of ultrasound processing on the rheological properties of milk such as viscosity, flow behavior index and consistency coefficient have not yet been reported and thus need to be investigated.

1.8. Lightness value of Milk

Extreme high temperature processing of milk such as ultra-high temperature (UHT) processing induces Maillard reactions, which can lead to color changes (Elliott et al., 2005; van Boekel et al., 1998). The appearance of milk can be measured on the basis of tristimulus

parameters in the L*a*b* mode. L* values range from 0 (black) to 100 (white) and represent lightness/reflectance of milk, which is related to the casein micelles in milk. Dissociation of micelles results in lower L-values because of decreased micellar size and thus the ability of milk to scatter light (Huppertz et al., 2002; O'Sullivan et al., 2002). A few published works have reported a decrease in the lightness value because of high pressure processing in skim milk (Johnston, 1995), raw milk (Mussa and Ramaswamy, 1997) and fresh raw bovine milk of 3.8% fat content (Bilbao-Sainz et al., 2009). Lightness value of high pressure processed milk was less than that of pasteurized milk (Pereda et al., 2007). Effects of ultrasound treatment on the color properties of milk have not yet been published. In order to determine the potential of ultrasound in fluid milk processing, it is imperative to investigate the effect of ultrasound processing at various treatment conditions on the color values of fluid milk and cream.

1.9. Particle Size Analysis

Fat globules in fluid milk are in the form of droplets whose diameter varies in the range of 0.2 to 20 μm (averaging around 4 μm) depending on cow breed and season (Mulder and Walstra, 1974). Homogenization, a mechanical treatment, disrupts milk fat globules, causing a decrease in their size and leading to uniform distribution of fat globules. Homogenization prevents separation of milk fat or creaming over time. Wu et al. (2001) showed that high power ultrasound (450W) is capable of homogenizing fluid milk better than commercial homogenization process. Villamiel and de Jong (2000) demonstrated that thermosonication at higher temperatures (between 70 and 75°C) resulted in a better particle distribution. Studies published studies on fat globule size of sonicated milk over storage time are scarce and thus we decided to study the effect of thermosonication on the size of milk fat globule over a time period of 30 days.

1.10. Research Objectives

Although ultrasound has a large potential for application in the dairy industry, the technique is not currently used by the industry for processing and/or preservation purposes because concrete data on the effects of ultrasound on shelf-limiting enzymes, sensory and other

quality parameters, energy consumption, scale-up etc. are limited. Our research objectives are to investigate the:

- effects of thermosonication (heat and ultrasound combined) at various amplitudes and treatment periods on the activity of total native plasmin and activity of bacterial (*Staphylococcus aureus V8)* protease over a period of four weeks
- odor profile of thermosonicated skim milk and cream over a period of four weeks
- effects of thermosonication on the color values and rheological properties of fresh skim milk and cream over a period of four weeks
- effects of thermosonication on the fat globule size of fresh skim milk and cream over a period of four weeks
- effects of thermosonication on the microflora of fresh skim milk and cream over a period of four weeks

1.11. Hypotheses

We hypothesize that thermosonication

- causes a significant reduction in the activity of total native plasmin and of *Staphylococcus aureus V8* protease
- does not significantly affect the odor profile of fresh skim milk and cream
- does not significantly affect the viscosity of fresh skim milk and cream
- does not significantly affect the color values of fresh skim milk and cream
- does not significantly affect the fat globule size of fresh skim milk and cream
- completely inactivates coliforms in fresh skim milk and cream
- causes a significant reduction in spoilage microflora of fresh skim milk and cream

CHAPTER 2. MATERIALS AND METHODS

2.1. Experiments

Preliminary study was conducted using store-bought pasteurized skim, reduced-fat and whole milk samples. Following the preliminary studies, further experiments were conducted using fresh, raw skim milk and cream samples (separated from fresh, raw whole milk) twice (hereafter called "Experiment I" and "Experiment II") with different treatments and type of analyses. Table 1 summarizes the experimental design showing the type of samples, treatments applied and type of analyses performed on the treated samples.

Table 1. Summary of experimental design

Section	Samples	Pre-treatment	Treatments	Analyses
PRELIMINARY STUDY	Commercial pasteurized skim (control), reduced fat (control) and whole milk (control)	Heated to 60°C	107 µm (60, 120, 150s) 119 µm (60, 120, 150s) 130 µm (60, 120, 150s)	*S. aureus* protease activity, viscosity and odor profile on day one (D1)
EXPERIMENT I	Raw skim milk (control) and cream (control) separated from raw ISU Dairy whole milk	Heated to 60°C	107 µm (60, 120, 180s) 133 µm (60, 120, 180s) 152 µm (60, 120, 180s)	Total plasmin activity, L* value and odor attributes (QDA) on D1, D15 and D30
EXPERIMENT II	Raw skim milk (control) and cream (control) separated from raw ISU Dairy whole milk	Heated to 65°C	107 µm (60, 120, 180s) 133 µm (60, 120, 180s) 152 µm (60, 120, 180s)	Total plasmin activity, L* value, and microflora on D1, D15 and D30; viscosity and fat globule size on D6, D15 and D30

2.2. Procurement of Samples and Chemicals

For preliminary experiments on *Staphylococcus aureus* protease, pasteurized skim, reduced-fat and whole milk (Anderson Erickson Dairy, Inc. Des Moines, IA) were purchased from a local grocery store. Fresh raw whole milk for experiments I and II on total native plasmin assay was procured from the bulk tank of Iowa State University's Dairy farm (Ames, IA). Whirl-Pak® sampling bags of 60 mL capacity were purchased from Sigma-Aldrich (St. Louis, MO) to store milk samples for various analyses. Sulfuric acid for Babcock test for fat content determination was purchased from LabChem, Inc. (Pittsburg, PA). Protease enzyme from *S. aureus V8,* bovine plasmin, ethylenediaminetetraacetic acid (EDTA) and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Fair Lawn, NJ). Azo-casein and tricholoroacetic acid for protease assay were obtained from Sigma-Aldrich (St. Louis, MO). Trizma buffer (pH 7.4), NaCl, H-D-valyl-Lleucyl-L-lysine-p-nitroanilide dihydrochloride (catalog number V7127), ε-amino caproic acid (ε-ACA; catalog number. A2504), and urokinase (catalog number U8627; EC 3.4.21.31) for total plasmin assay and plate count agar medium and violet red bile agar medium for microbial analysis were procured from Sigma-Aldrich (St. Louis, MO). Disposable UV methacrylate cuvettes (Fisherbrand) of 10mm path length and 1.5mL capacity for reading absorbance were purchased from the Iowa State University's Chemistry Stores (Ames, IA). Thinwall, Ultra-Clear™ ultracentrifuge tubes (14 x 95 mm) were purchased from Beckman Coulter (Brea, CA).

2.3. Sample Preparation

2.3.1. Preliminary Study

Pasteurized skim, reduced-fat and whole milk samples were purchased and stored at 4±1°C. Thermosonication was performed within 2 h of purchase and treated samples were stored in nalgene bottles and stored at 4±1°C until analyses.

2.3.2. Experiments I & II

Raw whole milk was stored at 4±1°C immediately after collection and was separated into skim milk and cream within 1 h of procurement. A centrifugal cream separator (1750 rpm)

from the Center for Crops Utilization Research pilot plant of Iowa State University was used for separation. The separator consists of 20 rotating discs that separate the whole milk into skim milk and cream fractions by means of centrifugal force. Raw skim milk and cream samples were collected in individual containers, labeled and refrigerated immediately. All samples were treated by thermosonication within 4 h of separation (experiment I) or thermosonication and heating (experiment II) within 8 h of separation and stored in sample glass vials (experiment I) sterile whirl-pack bags (experiment II) at 4±1°C. Samples for odor evaluation were stored in nalgene bottles coded with 3 digit random codes.

2.4. Analysis of Fat Content

2.4.1. Experiment I

Fat content of the raw whole, skim milk and cream from the first milk collection were determined using the Babcock method (AOAC, 2005). Milk and cream samples were tempered to 38°C and 17.6 ml of the tempered sample was accurately pipetted into appropriate size Babcock bottle. To this, 17.5 ml of reagent grade sulfuric acid (specific gravity, 1.82) was slowly added along the sides of the Babcock bottle's neck. The bottle was slowly swirled till all the curd in the bottle was dissolved. The Babcock bottles were placed in a centrifuge heated to 60°C and centrifuged for 5min. To the centrifuged bottles, soft water at 60°C was added until the liquid level was within 0.6 cm of the neck. The bottles were again centrifuged for 2 min and hot water was added near the top graduation level. The Babcock bottles were placed in a water bath at 57±0.5°C for at least 5 min to let the solution inside the Babcock bottles reach equilibrium. The bottles were then removed from the water bath, wiped, and the level of the fat in the graduated neck was read to the nearest 0.05%.

2.4.2. Experiment II

The fat, solids non-fat (SNF) and protein content of raw, heated and thermosonicated milk and cream samples from 2nd milk collection were determined using Lacticheck Milk Analyzer (Page & Pedersen Intl. Ltd., Hopkinton, MA). Approximately 20 mL of sample was taken in a small beaker, tempered to 16±1°C and fed into the sampling port for measurement. The percentage of fat, SNF and protein is directly read from the display. The equipment is

not calibrated for measuring fat contents higher than 9.0%. Thus cream samples were diluted with skim milk in the ratio of 1:3 before feeding the sample into the sample port.

2.5. Thermosonication Treatments

Thermosonication was performed using a batch sonicator (Brason Ultrasonics 2000 series), which supplied a power of approximately 400 W and generated ultrasound of 20 kHz frequency. A 1:1 booster and a 1:8 sonicating horn were used. Percentage of power supply to the horn was adjusted accordingly to vary the amplitude of the ultrasound waves to be dissipated. Table 2 shows the amount of power supplied and the corresponding amplitude of the ultrasound waves generated. The sonication vessel used was a jacketed pyrex glass chamber with loops at the bottom, which led to the increase in temperature of the sample that was sonicated. The sonicating horn was clamped and inserted into the sample with the tip of the horn reaching up to the level of sampling port/outlet. Approximately 100 mL of the sample was heated (to 60°C in the preliminary study and experiment I or 65°C in experiment II) on a hot plate heater. The open end of the container was sealed with aluminum foil and the sample was slowly stirred using a magnetic stirrer to ensure homogenous heating. The heated sample was immediately (within 5 s) transferred to the sonication chamber and sonicated. Amplitudes ranging from 107 to 152 μm were used for sonication time periods ranging from 1 to 3 min (Table 2). A circulating water bath was connected to the entrance and exit ports of the sonication vessel using rubber tubing. Water, at 72±0.5°C, was circulated through the sonication vessel during sonication to ensure that the sample temperature was taken up to 72°C and held there for at least 15 s (to mimic pasteurization). All treatments were applied in random order and in duplicate.

Controlling the temperature during sonication is important because temperatures above commercial pasteurization can affect sensory properties of milk as well as cause whey protein denaturation. Sonicating skim milk for 15 min without temperature control could result in increase in temperature, reaching up to 95°C and leading to denaturation of whey protein (Nguyen and Anema, 2010). Trials were run to ensure that milk temperature did not drop below 60°C before the start of sonication or rise beyond 75°C during and at the end of

the sonication process. Table 3 summarizes the temperature profiling experiments performed before thermosonicating fresh skim milk and cream samples. Temperature profiling was not performed for the preliminary study or experiment I. For experiment II, in addition to raw and thermosonicated samples, skim milk and cream samples that were heated to 72°C on the hot plate, and held for 1 min and 3 min with stirring, were also analyzed. These samples served as temperature controls to enable us to consider sonication effects separately from temperature effects.

For the preliminary study on *S. aurues* protease, the pasteurized skim, reduced-fat and whole milk samples were inoculated with the enzyme of 50 mU/mL activity before thermosonication. For experiments I and II on total native plasmin, the raw skim milk and cream samples were not inoculated with any enzyme before thermosonication.

Table 2. Amount of power supplied and amplitude of ultrasound waves generated

Amount of power supplied, %	Amplitude of ultrasound waves generated, µm (peak to peak)
67	107
74	119
83	133
95	152

Table 3. Temperature profile of sample during thermosonication

Sonication time (s)	Temperature of sample when thermosonicated at	
	133 µm (peak to peak)	152 µm (peak to peak)
0	65.8°C	65.2°C
30	68.1°C	68.6°C
60	72.4°C	70.2°C
90	73.0°C	71.5°C
120	73.8°C	72.2°C
150	74.1°C	72.8°C
180	74.6°C	73.5°C

2.6. Bacterial Protease Assay

For the assay of *S. aureus* protease activity, the azo-casein method, which was developed by Christen and Marshall (1984) and validated by Bendicho et al. (2002), was used. Approximately 1 ml of the sample was combined with 1 mL of 1% azo-casein solution and incubated immediately at 35.5°C for 15 min. The reaction was stopped by adding 2 mL of 5% tricholoracetic acid solution. The mixture was then centrifuged at 3000 RPM for 5 min and the absorbance of the supernatant was read at 280 nm using a UV/Visible (DU 720 general purpose Beckman Coulter) spectrophotometer. Enzyme activity was calculated from a standard curve prepared using azo-casein as substrate and known concentrations of the enzyme in buffer (Figure 1).

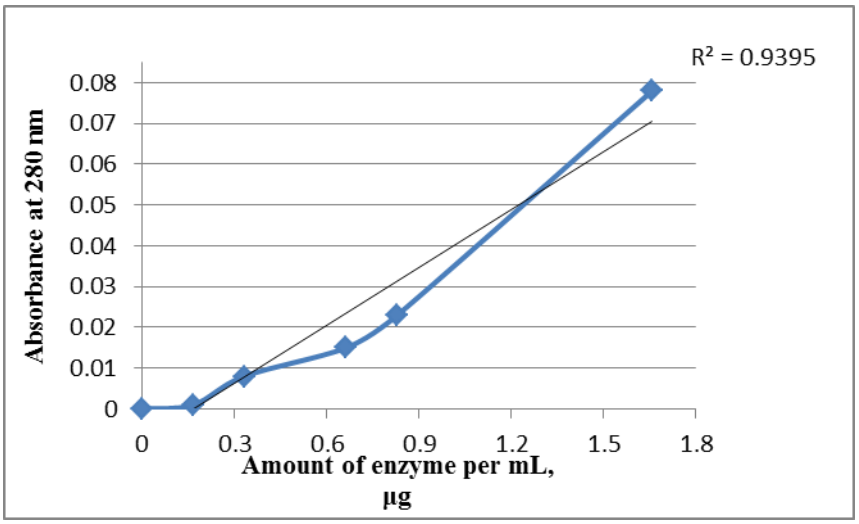

Figure 1. Standard curve prepared using *S. aureus* V8 protease

2.7. Total Plasmin Assay

Total plasmin activity is defined as the activity of plasmin determined after the conversion of inactive plasminogen to active plasminogen in the milk or cream samples. Total native plasmin activities in skim milk and cream samples were determined using the conventional methodology described by Politis et al. (1993) which is a modified version of the original method developed by Rollema et al. (1983). Approximately 5 mL of the sample was incubated with equal volume of 50 mM εACA for 2 h at room temperature. This allows the dissociation of the plasmin and plasminogen from the casein micelles and their transfer into the milk serum fractions. The treated sample was then ultracentrifuged using a Beckman L8-M Ultracentrifuge (Human Nutritional Sciences Building, Iowa State University, Ames, IA) at 100,000 X g for 1 h at 4°C to separate the milk serum and casein fractions. The top cream layer was discarded and the serum fraction was separated by decanting. The casein pellets were reconstituted to their original volume in 50 mM Tris buffer containing 110 mM NaCl. Approximately 50 µl of the milk serum or reconstituted casein was mixed with 950 µl of 50 mM Tris buffer containing 110 mM NaCl, 0.6 mM V7127 and 2.5 mM EACA. To this mixture, 150 plough units of urokinase were added, which started converting the inactive plasminogen to active plasmin in the mixture. The reaction mixture was incubated at 37°C for 1 h to allow sufficient conversion of the plasminogen (Korycka-Dahl et al., 1983; Politis

and Ng Kwai Hang, 1989). The absorbance of the reaction mixtures was read at 405 nm at 60-min interval for up to 20 h, and the absorbance vs. time curves were plotted. The rate of *p*-nitroanilide formation was calculated from the increasing linear portion of the curve. A similar reaction mixture without the milk serum or reconstituted casein fraction served as the blank. Enzyme activity was calculated from standard curve prepared using known concentrations of bovine plasmin (Figure 2). One unit of activity of plasmin plus plasminogen was defined as the amount of the enzyme that produced a change in absorbance at 405 nm of 0.001 in 1 min when *p*-nitroanilide was measured in the reaction mixture (Korycka-Dahl et al., 1983). Percentages of total plasmin activity of the sonicated samples were calculated based on the total plasmin activity of the initial raw skim milk and cream sample.

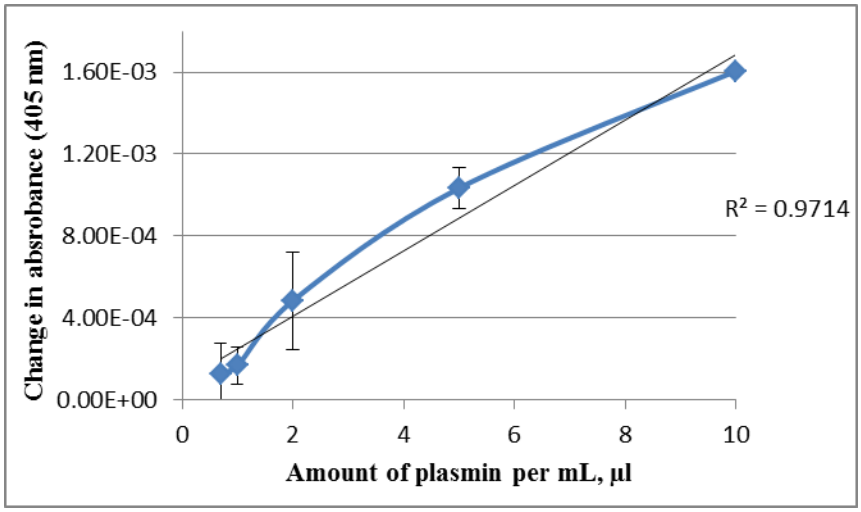

Figure 2. Plasmin standard curve prepared using bovine plasmin

2.8. Evaluation of Odor Profile

At an early stage of the project, pasteurized skim, reduced-fat and whole milk samples thermosonicated at 133µm for 2.5 min were subject to odor analysis by two expert dairy judging panelists, with over 30 hours of training in the evaluation of milk quality. The panelists evaluated the thermosonicated samples for the intensities of plastic/rubbery/burnt

aroma and cooked custardy aroma. The panelists also indicated the consumer acceptability of the thermosonicated samples in terms of their odor profile.

2.9. Descriptive Analysis

2.9.1. Panel Recruitment

Following the preliminary odor analysis, descriptive analysis of odor attributes of thermosonicated raw skim milk and cream was conducted. Approval for the use of human subjects in the study was obtained from the Institutional Review Board of Iowa State University. Panelists were recruited by posting ad-flyers around the university campus. The participants were required to drink milk at least once a month, have no aversions for odor associated with dairy products and must have interest in sensory evaluation. No preference on sex, nationality, smoking etc. was expressed. A total of 8 panelists were recruited, who were students and/or employees of Iowa State University.

2.9.2. Training Sessions

A total of 10 one-hour training sessions were conducted over a period of 5 weeks. During the 1st week, the panelists were introduced to sensory evaluation and its purpose and were given a brief description of the study and the objectives. Samples of pasteurized skim milk, whole milk and heavy whipping cream (Anderson Erickson Dairy, Inc. Des Moines, IA) obtained from the local grocery store were used for sniffing on the 1st session and the panelists were familiarized with the odors associated with commercial fluid milk and cream. At the end of the first session, odor detection ability of the panelists was determined using a triangle test. Samples used during the day's training session were used for the triangle test and each panelist evaluated a set of trio and identified the odd sample. One of the panelists dropped out after the first session because of schedule conflicts and was replaced by another panelist from the second training session. During the 2nd session, the panelists were asked to write down the odor attributes that they perceived in fresh, heated and thermosonicated (heated to 60°C and sonicated at 107 µm for 1 min and 152 µm for 3 min) pasteurized skim, reduced-fat and whole milk and heavy whipping cream. A list of the terms that the panelists came up with initially is listed in Table 3. During the next four consecutive sessions, the descriptor

terms for four major odor attributes were short-listed from the initial list after the panelists arrived to a consensus. This was accomplished by comparing the odor profile of reference samples for the various attributes generated initially with that of the fresh and thermosonicated milk and heavy cream samples. Terms that seemed to be redundant, too mild to be detected by most other panelists, coined by misconception of their characteristic or without confidence were removed from the list. The term flat implied that the sensory attribute is almost nil in intensity and thus it was removed from the list. Terms such as bleach, plastic, cardboard, soapy, beany, fishy, burnt hair, cabbage were removed because the panelists did not perceive these attributes when they were provided with the same type of references during the third and fourth sessions. To demonstrate the difference between scalded and cooked aroma, samples of pasteurized skim milk bought from the store and heated on stove-top were provided for sniffing. Samples were removed when the milk reached the temperatures: 45°C, 60°C, 75°C, 85°C and near boiling. The panelists agreed that the milk that was sampled out near boiling was scalded whereas the ones that were sampled out at 75°C and 85°C had cooked aroma. The panelists also agreed to the fact that the cooked and scalded milk samples had nutty and eggy notes in their odor profile and need not have to be considered as separate descriptor terms. Hence the terms ‘cooked’ and ‘scalded’ were removed from the list. The panelists considered that the term ‘custardy’ was to ‘eggy’ because the aroma of custard closely resembled that of hard boiled eggs but with a lesser intensity (7.5 on a 15 cm scale). Thus custardy was removed from the list of descriptors. Also, the sweet aroma associated with fresh custard was considered to be on nutty scale with an intensity of 3.5 and thus sweet-aromatic was removed from the list as well. Vanilla and corn meal were removed from the list because the panelists associated them with the sweet aromatic nature which comes under the nutty attribute. At the end of the fifth session it was decided that four odor descriptors, eggy, nutty, rubbery and creamy terms, would be the attributes the panelists would be evaluating in the evaluation sessions. The final list of descriptor terms, along with the reference samples used to anchor panelists, is shown in Table 4. During anchoring, the panelists compared the odor profile of milk and cream samples (fresh, pasteurized and thermosonicated) with the reference samples and decided the samples that would corresponded to the maximum and minimum intensities of each major

attribute - eggy, nutty, rubbery and creamy. The aroma of fresh hard boiled eggs was decided to be the maximum intensity (15 point on a 15 cm line scale) of eggy odor attribute whereas that of fresh custard and 2 day's old custard were considered to be of intensity 7.5 and 2 on the 15 cm line scale. Fresh custard was also considered to have a nutty odor of intensity 3.5. The panelists expressed that the pecan nuts toasted for 3 min possessed the maximum intensity of nutty aroma whereas those toasted for 1 min had a nutty odor intensity of 10 on the line scale. Elastic rubber bands were provided to compare with the rubbery odor attribute of milk and cream samples. The panelists expressed that odor of those rubber bands would be the strongest intensity and thus were placed on the maximum end of the line scale. For creamy attribute, store bought pasteurized heavy-cream samples were considered to possess the maximum intensity of creamy odor whereas pasteurized whole milk was considered to have an intensity of 5 on creamy odor line scale. In all cases pasteurized skim milk fell on the weakest end of the odor intensity because of the flat nature of its aroma profile. The panelists considered pasteurized skim milk to fall on the 15 cm line scale at point 1 for eggy and creamy attributes and at 2 for rubbery attribute. The panelists did not associate pasteurized skim milk with nutty odor. A list of the preparation methods for the samples that were used for the generation and narrowing down of the odor descriptors, as well as for the training sessions, are provided in Table 6. All the samples were refrigerated after preparation and were tempered to approximately 10±1°C before the training session. The panelists were provided with descriptor sheets with 15 cm line scales to mark the intensities of each odor attribute, remembering the scores of the reference samples used during the training sessions.

Table 4. Initial list of odor descriptors coined by the panelists

Pasteurized		Pasteurized and heated		Pasteurized and thermosonicated	
Skim milk	**Cream**	**Skim milk**	**Cream**	**Skim milk**	**Cream**
Flat	Eggy*	Plastic	Beany	Flat	Fishy
Bleach	Custardy	Creamy*	Vanilla	Eggy	Custardy
	Cooked	Cooked	Nutty*	Plastic	Burnt hair
		Cardboard		Sweet-aromatic	Rubbery*
		Soapy		Corn meal	Plastic
		Scalded			Cabbage

* Attributes selected in the final list of major odor attributes

Table 5. References used for training panelists on varying intensities of odor attributes

Odor descriptor	Reference	Intensity of attribute
Eggy	Fresh hard-boiled eggs	15
	Fresh custard	7.5
	2 day's old custard	2
	Pasteurized skim milk	1
Nutty	Pecan nuts toasted for 3 min	15
	Pecan nuts toasted for 1 min	10
	Fresh custard	3.5
Rubbery	Elastic rubber bands	15
	Pasteurized skim milk	2
Creamy	Pasteurized heavy whipping cream	15
	Pasteurized whole milk	5
	Pasteurized skim milk	1

Table 6. Preparation of samples for training sessions

Sample	Purpose	Preparation method
Heated milk and cream samples	Introduction to odor attributes for coining of odor descriptors	Approximately 200 mL of the sample was microwaved on high for 2 min in a paper cup.
Jell-O - *cook and serve* custard dessert mix	Reference sample for narrowing down the descriptors	The dessert mix (58 g) was stirred in 2 cups of reduced-fat milk in a microwavable bowl and microwaved on high for 6 min, when the mixture came to a full boil. The mixture was stirred after 4 min.
Fresh custard	Reference sample for eggy attribute (intensity score: 7.5)	Two cups of pasteurized reduced-fat milk was boiled to scalding. Two egg yolks were beaten and mixed with 1/3rd cup of sucrose and this was slowly stirred into scalded milk under low heat. After the mixture thickened, it was removed from heat and chilled over night in refrigerator.
Hard boiled eggs	Reference sample for eggy attribute (intensity score: 15)	Grade A fresh eggs were boiled in water, de-shelled and quartered
Toasted pecan nuts (1min and 3min)	Reference sample for nutty attribute	Pecan nuts were toasted on iron skillet for 1 min (intensity score: 10) 3 min (intensity score: 15)
Thermosonicated skim milk and cream	Reference sample for narrowing down the descriptors	Pasteurized samples were obtained from local stores, heated to 60°C, and sonicated at 152 µm for 5min.

2.9.3. Sniffing Procedure

A standard procedure for sniffing the samples was followed by all panelists. The glass sample containers of 30 mL volume (U.S. Plastic Corp) with lids closed were placed on the palm and enclosed by fingers. The container was then slowly swirled for approximately 20 s.

Shaking the containers was not recommended because of possibility of foam formation that might interfere with sniffing. After warming up the sample by swirling, the lids were opened and the sample was evaluated by a deep sniff with nose close to the brim of the container. Not more than two or three sniffs were recommended for evaluating a sample in order to avoid fatigue arising from continuous sniffing.

2.9.4. Evaluation Sessions

After 10 h of training, the panelists evaluated the specific odor attributes of thermosonicated skim milk and cream samples. We wanted to evaluate the effect of extreme thermosonication treatment conditions on the odor attributes and wanted to avoid fatigue rising from sniffing too many samples. Thus we used the samples that were thermosonicated at 152 μm for 3 min only. Fresh pasteurized commercial samples of skim milk and heavy-whipping cream served as controls and were provided for sniffing on the same days when the treated samples were evaluated. The panelists were allowed to use notes to help them recall samples and scores for the various attributes during the evaluation sessions. Further elastic rubber bands and pecan nuts toasted for 3 min, which served as reference samples for rubbery and nutty aroma attributes, were provided in the booths to the panelists for anchoring themselves once every two weeks prior to evaluating the samples. Panelists evaluated the samples in triplicates for a storage period of up to 4 weeks for both skim milk and cream. Because of a large number of samples (30, for skim milk and cream each), a balanced incomplete block design was followed to prevent the chances of fatigue arising from sniffing many samples. All the samples (including triplicates) were spread and served over a period of 5 weeks. Samples were served in a random order at all the sessions to reduce the error from bias and influence from other panelists, although the panelists were instructed not to discuss their opinions during the evaluation sessions. During each evaluation session, samples were freshly opened from nalgene bottles stored at 4°C and delivered to individual serving containers for sniffing. Approximately 15 mL of the sample was filled into 30 mL sample glass container (U.S. Plastic Corp.) such that each panelist had their own individual set of samples. The plastic lids of the container were labeled with randomly generated three-digit codes to hide the identity of the samples from panelists. Samples were served at a temperature of 10±1°C.

Skim milk samples were evaluated on Tuesdays while heavy cream samples were evaluated on Thursdays in order to be consistent with the days of evaluation for all the triplicate samples. Score sheets with panelist number and sample number were provided to each panelist, along with sample, water and paper napkin. The panelists followed the standard sniffing procedure and evaluated the samples by marking the intensity of the odor attribute on a 15 cm line scale (Figure 3).

Sensory Evaluation of Milk and Cream

Panelist # _____ **Sample #_____**

Line of Scale for Descriptors

1. Eggy

None _____ Strong

Comments

_____

2. Nutty

None _____ Strong

Comments

_____

3. Rubbery

None _____ Strong

Comments

_____

4. Creamy

None _____ Strong

Comments

_____

Figure 3. Evaluation sheet for the descriptive analysis of odor attributes

The panelists retained the same panelist number throughout the end of the study. In order to prevent the panelists from comparing samples, the sample and the score sheet were taken back from the panelist before the next sample for evaluation was served. The intensity of attributes marked by the panelists on the score sheets for samples was measured using a ruler and recorded.

2.10. Determination of Lightness Value

The lightness or reflectance (L*) value of the thermosonicated skim and heavy cream samples were measured instrumentally using a LabScan® XE spectrophotometer (Food Sciences Building, Iowa State University, Ames, IA) on days 0, 14, and 28. Approximately 20 mL of the fluid sample was poured into a transparent glass circular dish. The colorimeter was calibrated against white and black tile standards, and operated at a 10° angle of illuminant D65. All samples were tempered to 23±1°C before analysis. L* value indicates the lightness (0 - black; 100 - white) of the milk or cream sample. The glass dish was rinsed with deionized water and wiped dry between samples.

2.11. Determination of Viscosity

Viscosity of the samples was determined using a HAAKE RS150 Rheometer (Food Sciences Building, Iowa State University, Ames, IA). A double gap cylinder and DG41 sensor were used. Air pressure was maintained between 250000 and 300000 Pa. Shear rates of 0 to 1000 s^{-1} were used. Samples were tempered to room temperature of 23±1°C before analysis. The sensor was fixed to the rotor and zeroed with the cylinder. Approximately 6.3 mL of the sample was pipetted into the coaxial cylinder. The sensor was then taken to the default gap of 5.1 mm before it was allowed to start the rheological measurements. Rheological responses of the samples were modeled using HAAKE Rheowin Data Manager provided by Thermo Scientific to determine the viscosity of the sample and newton law model was used to fit the flow behavior of the milk samples.

2.12. Measurement of Fat Globule Size

Size of fat globules in raw and treated milk and cream samples were determined using Mastersizer 2000 particle size analyzer (Malvern Instruments, Malvern, UK), which uses laser light scattering technique. Laser obscuration rate was maintained between 13.2% and 18.0% and pump/stir speed was set at 2040 rpm. Sample beaker was filled with deionized water for flushing the tank thrice and for laser alignment. Deionized water was also used as the sample dispersant. The refractive indices of milk fat and deionized water are 1.45 and 1.33, respectively. Milk and cream samples were diluted in the ratio of 1:1 using 35 mM EDTA (pH adjusted to 7.0 using NaOH), which dissociates casein micelles from fat globules. The diluted sample is then dispersed into sample beaker filled with the dispersant for measurement. The diameter of the distribution peak, sauter diameter ($D_{3,2}$) and the volume-weighted diameter ($D_{4,3}$) were determined by the Malvern software. The specific area (S) was calculated from the sauter diameter [$S = 6/(\rho * D_{3,2})$], where ρ is the density of milk fat, which is 0.92 (Mulder and Walstra, 1974).

2.13. Microbiological Analysis

Microbiological quality of milk/cream samples were evaluated by enumerating total aerobic count (TAC) and coliform count in all experiment II samples. Raw samples were analyzed only on day one (within 24 h of D1), while heated and thermosonicated samples were analyzed on D1±24 h, D15 and D30.

2.13.1. Enumeration of Total Aerobic Bacterial Count

Plate count agar (Sigma-Aldrich) medium was used to enumerate TAC. Media was dissolved in deionized water using a magnetic stirrer on hot plate heater, boiled for 1 min, autoclaved at 121°C and 100 kPa for 15 min and cooled down to around 45±5°C before plating. Samples were diluted in 0.1% autoclaved peptone solution (Sigma-Aldrich) and plated using pour-plating method. During plating, an overlay method was employed to reduce the spreading of the colonies. Plates were incubated at 37°C and colonies were enumerated after 48 h. Total aerobic count was expressed as colony forming units (CFU) per mL.

2.13.2. Enumeration of Coliform Count

Violet red bile agar (VRB) medium was used to enumerate coliform count. Media was dissolved in deionized water on hot plate, boiled for 1 min and cooled to approximately 45±5°C. Samples were diluted in 0.1% peptone solution (autoclaved) and plated using pour-plating method. Plates were incubated at 37°C and colonies were enumerated after 24 h. Coliform count was expressed as CFU/mL.

2.14. Data Analysis

Analyses were performed in duplicate on individual milk/cream samples for all the experiments except for sensory evaluation, which was done in triplicate. Results were analyzed by Analysis of Variance using SAS (version 9.2) and Microsoft Excel 2007. A significance level of $\alpha = 0.05$ was used to determine the significant differences.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Proximate Analysis

The percentage of fat measured in the fresh raw whole milk obtained from the ISU Dairy and the separated raw skim milk and cream are listed in Table 7 (experiment I). Analysis was performed in triplicate for two collection dates.

Table 7. Fat content of raw whole milk, raw skim milk and cream fractions (Experiment I)

Sample	Fat content (mean ± SD), %
Raw whole milk	3.60±0.20
Raw skim milk	0.53±0.12
Raw cream	45.50±2.18

The average fat content of raw whole milk was 3.60%, which is similar to the value claimed by the ISU Dairy. According to title 21 of Code of Federal Regulations skim milk should contain less than 0.5% of fat (Code of Federal Regulations, 2011a) and heavy cream should contain at least 36% of fat (Code of Federal Regulations, 2011b). The raw cream fraction separated from the raw whole milk meets the CFR standards. Even though the raw skim milk fraction appears to be slightly higher in fat content than the standard for skim milk drawn by CFR, the difference was considered to be negligible. Also, no further efforts to obtain skim milk of fat content less than 0.5% were attempted because of equipment limitations.

Table 8 shows fat, SNF and protein content values of whole milk and Table 9 shows those of milk and cream samples from experiment II milk collection, measured using Lacticheck Milk Analyzer. Fat content of thermosonicated skim milk samples were non-significantly higher ($p>0.05$) than that of raw and heated skim milk samples (Table 9). This could be because of the cavitation intensity, which resulted in breakage of fat globules (homogenization) making the butter fat more available for analytical quantification and thus a slightly higher fat content was read by the Lacticheck milk analyzer. Our results are in agreement with that of

Bermudez-Aguirre et al. (2009) who reported that thermosonication of whole milk resulted in increased fat content.

Table 8. Proximate analysis of raw whole milk (Experiment II)

Component	Content (mean±SD), %
Fat	4.36 (±0.13)
SNF	9.69 (±0.10)
Protein	3.66 (±0.04)

Table 9. Proximate analysis of raw and treated milk and cream samples on D1±24 h (Experiment II)

Sample	Fat (mean ± SD), %		SNF (mean ± SD), %		Protein (mean ± SD), %	
	Skim milk	Cream	Skim milk	Cream	Skim milk	Cream
Raw	0.73 ± 0.07 [a]	19.91 ± 1.56 [a]	10.35 ± 0.07 [a]	35.79 ± 0.08 [a]	3.85 ± 0.01 [a]	12.19 ± 0.03 [a]
Heated to 72°C for 1 min	0.62 ± 0.05 [a]	20.83 ± 0.20 [a]	10.35 ± 0.07 [a]	35.37 ± 0.34 [a]	3.86 ± 0.04 [ab]	12.05 ± 0.11 [a]
Heated to 72°C for 3 min	0.70 ± 0.08 [a]	18.15 ± 0.65 [ab]	10.15 ± 0.07 [a]	35.99 ± 0.25 [a]	3.84 ± 0.02 [ab]	12.23 ± 0.08 [a]
TS at 133 µm for 1 min	0.72 ± 0.05 [a]	17.43 ± 0.20 [ab]	9.91 ± 0.27 [a]	34.45 ± 0.06 [a]	3.70 ± 0.09 [b]	11.67 ± 0.03 [ab]
TS at 133 µm for 3 min	0.72 ± 0.01 [a]	16.91 ± 1.67 [ab]	10.25 ± 0.07 [a]	35.03 ± 0.42 [a]	3.84 ± 0.03 [ab]	11.87 ± 0.14 [ab]
TS at 152 µm for 1 min	0.81 ± 0.02 [a]	17.57 ± 0.23 [ab]	10.10 ± 0.01 [a]	34.57 ± 0.28 [a]	3.77 ± 0.01 [ab]	11.73 ± 0.11 [ab]
TS at 152 µm for 3 min	0.77 ± 0.02 [a]	17.27 ± 0.65 [ab]	10.25 ± 0.07 [a]	34.65 ± 0.11 [a]	3.82 ± 0.01 [ab]	11.75 ± 0.03 [ab]

TS – thermosonicated; Means within the each column with the same letter are not significantly different.

3.2. Protease Assay

3.2.1. Bacterial Protease Assay

The initial activity of *Staphylococcus aureus V8* protease used for preliminary study thermosonication experiments was 50 mU/mL and the average activity levels of the bacterial protease (in percentage) after thermosonication of pasteurized skim, reduced-fat and whole milk media are listed in Table 10.

Table 10. Activity of *S. aurues V8* protease in pasteurized milk (Preliminary study)

Treatment	Average protease activity (mean ± SD), %		
	Pasteurized skim milk	**Pasteurized reduced-fat milk**	**Pasteurized whole milk**
TS at 107 µm for 1 min	184.00 ± 8.72^{a}	136.67 ± 4.16^{abcd}	122.00 ± 32.00^{bdec}
TS at 107 µm for 2 min	174.67 ± 13.61^{abcd}	98.67 ± 14.05^{gfdec}	110.00 ± 26.46^{gfeh}
TS at 107 µm for 2.5 min	143.33 ± 6.11^{gfeh}	68.67 ± 8.08^{gfeh}	80.00 ± 26.00^{gfih}
TS at 119 µm for 1 min	138.00 ± 13.11^{ab}	92.67 ± 3.06^{fdec}	77.33 ± 45.88^{fdec}
TS at 119 µm for 2 min	84.67 ± 13.32^{gfdech}	54.67 ± 6.11^{gfih}	60.67 ± 21.01^{gfih}
TS at 119 µm for 2.5 min	62.00 ± 2.00^{gfih}	30.00 ± 7.21^{ih}	37.33 ± 43.88^{gih}
TS at 133 µm for 1 min	69.33 ± 10.07^{abc}	74.67 ± 12.86^{gfeh}	54.67 ± 27.30^{gfdeh}
TS at 133 µm for 2 min	52.67 ± 14.19^{gfih}	26.67 ± 6.11^{ih}	34.67 ± 13.32^{gih}
TS at 133 µm for 2.5 min	38.00 ± 2.00^{gih}	8.67 ± 3.06^{i}	28.67 ± 3.06^{ih}

TS – thermosonicated; Means within the each column with the same letter are not significantly different.

Overall, the F-Test showed significant differences in the enzyme activity because of sonication amplitude, and sonication time, and there were interaction effects in all the three media ($p<0.05$).

In pasteurized skim milk medium, thermosonication at 107 and 119 µm for 1 min resulted in an increase in enzyme activity compared to that of untreated samples. Further sonication resulted in a decrease in enzyme activity. Similarly, in pasteurized reduced-fat milk medium

and whole milk medium, thermosonication at 107 µm for 1 min resulted in an increase in enzyme activity from that of untreated samples and further sonication resulted in a decrease in enzyme activity. The initial increase in enzyme activity could be because of substrate activation (Sakakibara et al., 1996) or increased rate of conversion of plasminogen to plasmin. Researchers have reported that mild ultrasonication can increase the activity of free enzymes (Özbek and Ülgen, 2005; Sakakibara et al., 1996). Sonicating for 2.5 min at 107, 119 and 133 µm resulted in reduction of enzyme activity by 21.4%, 16.0% and 72.0%, respectively in skim milk medium, by 32.0%, 70.0% and 92.0% respectively in reduced-fat milk medium and by 21.4%, 72.0% and 72.0% respectively in whole milk medium.

Maximum inactivation of the enzyme rate was achieved in reduced-fat milk medium followed by whole milk and skim milk media. Researchers have reported that the presence of fat in the medium can protect protein molecules from denaturation because of cavitation (Dumay et al., 1994; Murray, 1997); however, our data doesn't seem to follow this theory and thus the relation between fat content and *S. aureus* protease inactivation by thermosonication could not be determined.

Lower sonication amplitudes of 107 and 133 µm increased the average activity of the bacterial enzyme in pasteurized skim milk when sonicated for 60 s. Similarly, sonicating at 107 µm for a time period of 60 s increased the activity of the enzyme in pasteurized reduced-fat and whole milk, which then dropped down with an increase in treatment amplitude and/or time. The initial increase in enzyme activity could be because of the fact that ultrasonication increases mass flow rate, thus enabling faster transport of substrate molecules to the enzyme molecules (Mason et al., 1996). Further increase in treatment time and/or amplitude caused a decrease in enzyme activity in both cases and this could be because of increase in cavitation intensity in the sonicating medium.

Research has shown that ultrasound acts synergistically with heat to inactivate enzymes in food systems. The postulated theory is that higher temperatures can yield unfolding of the native structure of the enzyme and thus increase the efficiency of the inactivation mechanism of ultrasound (Lopez et al., 1994; Lopez et al., 1998; Vercet et al., 1999). Researchers

reported that ultrasonication at 120 µm, after heating to 61°C, decreased the activity of alkaline phosphatase by approximately 49% in whole milk and 44% in skim milk, whereas conventional heating at 70°C decreased the enzyme activity by only 24% in both skim and whole milk (Villamiel and de Jong, 2000). The authors also reported that there is complete inactivation of the enzyme when sonicated at 75.5°C for 102.3 s. Further, the free radicals formed because of pyrolysis can interact with enzymes and can cause oxidative modifications of amino acids in the active site. In addition to these modifications, the free radicals can also cause denaturation, covalent aggregation, and fragmentation reactions on the enzymes (Davies, 1987a; Davies, 1987b; Davies 1987c; Hunt et al., 1988). These could be the reactions that cause the decrease in activity of *S. aureus* protease during thermosonication.

3.2.2. Total Plasmin Assay

The initial total plasmin activity values of raw skim milk and cream for experiments I and II are reported in Table 11. Significant differences were observed in plasmin activity between raw skim milk and cream fractions ($p<0.05$) and the average plasmin activity of raw cream fraction was found to be approximately twice that of raw skim milk fraction separated from the raw whole milk used for experiment 1. The higher plasmin activity found in cream could possibly be because of the plasminogen associated with milk fat globules in the cream matrix (Politis et al. 1992).

Table 11. Total Plasmin Activity in raw skim milk and cream fractions evaluated in experiments I and II

Fraction	Total Plasmin Activity (mean ± SD), mU/mL	
	Experiment I	Experiment II
Skim milk	14.70 ± 0.57	17.64 ± 1.30
Cream	27.60 ± 3.39	26.13 ± 5.07

The average total plasmin activity values after thermosonication of skim milk and cream (experiment 1) are listed in Table 12. In raw skim milk, thermosonication at 107, 133 and

152 µm decreased the total plasmin activity by approximately 76.79%, 83.24%, and 80.9%, respectively when sonicated for 3 min. The average total plasmin activity of these samples measured on day 28 was approximately two-folds higher than that on day 0. This could be because of the refolding of the inactivated enzyme back to its native state, which enabled the enzyme to become active slowly with time. Researchers have reported that plasmin is a robust enzyme with a high conformational stability. Even though plasmin readily unfolds upon heating, the unfolded enzyme is resistant to degradative reactions and upon cooling the unfolded enzyme refolds into its active state again (Daniel, 1996). Some researchers have associated inactivation of plasmin in milk to the presence of β-lactoglobulin (β-lg). When heated in the absence of β-lg, the thermally unfolded plasmin will refold into its active state i.e. plasmin is very heat resistant in the presence of β-lg. The low plasmin activity in UHT milk is attributed to the thermal denaturation of β-lg (Hui, 2006). Denaturing β-lg by heat exposes the reactive sulfydryl group, which in turn undergoes disulfide-sulfydryl interchange reactions with the disulfide bonds of plasmin; this can have a destabilizing effect on plasmin i.e. the heterologous disulfide-line complexes prevent refolding of plasmin and thus inhibit its activity. Researchers have shown different results on the influence of state of β-lg on plasmin activity. Bastian et al. (1993) reported that native β-lg inhibited plasmin activity against casein more than denatured β-lg (Bastian et al., 1993), whereas Rollema and Poll (1986) reported that denatured β-lg had more inhibitory effect on casein than native β-lg (Rollema and Poll, 1986). The differences in the results could be attributed to the differences in experimental design such as type of substrate, heat treatment given to samples prior to assay etc. From Table 11, it can be seen that the total plasmin activity values of the treated cream samples were significantly less than those of the untreated, raw cream sample. In raw cream, thermosonication at 107, 133 and 152 µm decreased the total plasmin activity by approximately 85.67%, 84.17%, and 96.67%, respectively when sonicated for 3 min. The average total plasmin activity for most of the samples was observed to decrease with storage time, unlike in skim milk, where the activity was found to increase over storage time. The difference in behavior of plasmin activity of thermosonicated skim milk and cream could also be because of the presence of plasminogen inhibitors and activators. Conventional pasteurization inactivates plasminogen inhibitors while the plasminogen activators are heat-

stable. Thus, studies on the effect of treatments on plasminogen activators and inhibitors would help to explain the increase and decrease in total plasmin activity in skim milk and cream samples, respectively, upon storage.

Table 12. Average total plasmin activity in skim milk and cream (Experiment I)

Treatment	Average total plasmin activity (mean ± SD) of skim milk, %			Average total plasmin activity (mean ± SD) of cream, %		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Raw	100.00 ± 3.85 $^{a\,b\,c}$	--	--	100.00 ± 12.30 ^{a}	--	--
TS at 107 µm for 1 min	61.73 ± 8.63 $^{b\,d\,c}$	67.12 ± 5.95 $^{b\,d\,c}$	179.19 ± 14.59 ^{a}	18.33 ± 0.47 $^{b\,c}$	8.17 ± 1.18 ^{c}	7.50 ± 0.24 ^{c}
TS at 107 µm for 2 min	26.84 ± 1.37 $^{d\,c}$	19.37 ± 0.70 $^{d\,c}$	34.15 ± 7.09 $^{b\,d\,c}$	16.00 ± 0.30 $^{b\,c}$	8.67 ± 0.47 ^{c}	4.17 ± 0.24 ^{c}
TS at 107 µm for 2.5 min	22.12 ± 0.37 $^{d\,c}$	14.30 ± 0.55 $^{d\,c}$	24.07 ± 13.43 $^{d\,c}$	14.00 ± 1.41 ^{c}	12.67 ± 1.89 ^{c}	2.83 ± 0.24 ^{c}
TS at 107 µm for 3 min	23.51 ± 2.35 $^{d\,c}$	3.47 ± 1.65 $^{d\,c}$	64.18 ± 12.09 $^{b\,d\,c}$	14.33 ± 4.71 $^{b\,c}$	6.67 ± 2.83 ^{c}	3.83 ± 0.71 ^{c}
TS at 133 µm for 1 min	26.68 ± 5.79 $^{d\,c}$	16.67 ± 0.16 $^{d\,c}$	30.08 ± 7.89 $^{d\,c}$	20.50 ± 1.18 $^{b\,c}$	6.00 ± 0.47 ^{c}	9.00 ± 0.47 ^{c}
TS at 133 µm for 2 min	11.78 ± 1.13 $^{d\,c}$	14.21 ± 0.51 $^{d\,c}$	30.63 ± 1.18 $^{d\,c}$	13.00 ± 0.47 ^{c}	6.83 ± 1.65 ^{c}	13.57 ± 0.77 ^{c}
TS at 133 µm for 2.5 min	28.59 ± 1.10 $^{d\,c}$	17.77 ± 4.53 $^{d\,c}$	42.80 ± 14.63 $^{b\,d\,c}$	13.33 ± 1.89 ^{c}	4.17 ± 2.12 ^{c}	7.33 ± 0.94 ^{c}
TS at 133 µm for 3 min	16.76 ± 4.97 $^{d\,c}$	3.77 ± 1.59 $^{d\,c}$	30.54 ± 3.63 $^{d\,c}$	15.83 ± 0.71 $^{b\,c}$	4.50 ± 0.71 ^{c}	10.33 ± 0.47 ^{c}
TS at 152 µm for 1 min	27.56 ± 0.58 $^{d\,c}$	16.20 ± 0.43 $^{d\,c}$	23.79 ± 1.01 $^{d\,c}$	14.00 ± 3.77 ^{c}	7.33 ± 0.94 ^{c}	3.33 ± 0.94 ^{c}
TS at 152 µm for 2 min	27.89 ± 0.11 $^{d\,c}$	7.92 ± 0.32 $^{d\,c}$	45.82 ± 12.35 $^{b\,d\,c}$	17.77 ± 2.31 ^{b}	13.17 ± 0.71 ^{c}	2.83 ± 0.24 ^{c}
TS at 152 µm for 2.5 min	25.30 ± 6.75 $^{d\,c}$	4.25 ± 0.08 $^{d\,c}$	27.69 ± 7.32 $^{d\,c}$	10.17 ± 0.71 ^{c}	3.67 ± 0.47 ^{c}	3.33 ± 0.94 ^{c}
TS at 152 µm for 3 min	19.10 ± 2.66 $^{d\,c}$	1.84 ± 0.26 $^{d\,c}$	37.77 ± 0.97 $^{b\,d\,c}$	3.33 ± 3.77 ^{c}	3.67 ± 0.20 ^{c}	8.83 ± 1.18 ^{c}

TS – thermosonicated; Means with the same letter within skim milk/cream columns are not significantly different.

Table 13. Average total plasmin activity in skim milk and cream (Experiment II)

Treatment	Average total plasmin activity (mean ± SD) of skim milk, %			Average total plasmin activity (mean ± SD) of cream, %		
	D0	D15	D30	D0	Day 15	D30
Raw	100 ± 24.05 ^{bc}	--	--	100 ± 26.42 ^{a}	--	--
Heated to 72°C for 1 min	75.59 ± 5.34 ^{bcd}	24.06 ± 6.00 ^{efd}	309.98 ± 42.65 ^{a}	36.76 ±7.08 ^{bcd}	34.69 ± 13.28 ^{bcd}	44.06 ± 9.91 ^{bc}
Heated to 72°C for 3 min	49.13 ± 1.69 ^{cdef}	25.06 ± 2.01 ^{efd}	109.87 ± 20.99 ^{b}	12.35 ± 3.02 ^{cd}	26.69 ± 6.98 ^{cd}	70.91 ± 17.32 ^{ab}
TS at 133 µm for 1 min	13.23 ± 8.02 ^{ef}	19.81 ± 4.01 ^{efd}	65.19 ± 12.03 ^{bcde}	4.10 ± 1.16 ^{d}	8.72 ± 0.44 ^{cd}	4.97 ± 0.64 ^{d}
TS at 133 µm for 3 min	5.67 ± 2.67 ^{f}	12.74 ± 2.10 ^{ef}	25.51 ± 12.02 ^{efd}	3.28 ± 4.23 ^{d}	3.16 ± 0.64 ^{d}	3.62 ± 1.28 ^{d}
TS at 152 µm for 1 min	26.46 ± 5.34 ^{efd}	28.30 ± 8.01 ^{efd}	102.04 ± 16.03 ^{bc}	2.46 ± 1.16 ^{d}	5.87 ± 1.91 ^{d}	5.88 ± 1.92 ^{d}
TS at 152 µm for 3 min	5.67 ± 2.67^{f}	4.25 ± 2.13 ^{f}	51.02 ± 16.03 ^{cdef}	5.74 ± 3.48 ^{d}	1.81 ± 0.55 ^{d}	5.42 ± 2.56 ^{d}

TS – thermosonicated; Means with the same letter within skim milk/cream columns are not significantly different.

In experiment 1, overall F-Test showed no significant differences in enzyme activity because of sonication amplitude in skim milk on day 0. However, significant differences were observed because of sonication time and an interaction of sonication time and amplitude in skim milk on day 0 ($p<0.05$). Also, strong significant differences because of interaction were observed among the enzyme activity on days 14 and 28 in skim milk samples ($p<0.01$). In cream samples, the overall F-Test showed no significant differences in enzyme activity on day 0. However, significant differences were observed in enzyme activity because of sonication time and interaction effect on day 14 and because of amplitude and interaction effect on day 28 ($p<0.05$). Similar results were observed in experiment II.

Our results have shown that thermosonication was more effective in reducing the total plasmin activity in cream than in skim milk, thus implying that the fat content in milk and

cream may not have played a role in protecting the plasmin system from inactivation upon thermosonication.

In experiment 1, total plasmin activity in the skim milk for all the thermosonication treatments levels (amplitude and time) was lesser on day 14 when compared to day 1 except for the one treated at 107 µm for 60 s. Total plasmin activity for all the samples on day 30 was higher than that of day 14 showing partial to complete regeneration of their activity. Total plasmin activity of the samples on day 28 was approximately one to two folds higher than that of day 0. Total plasmin activity in cream samples thermosonicated at 107 µm decreased upon storage from day 0 to day 28 and those thermosonicated at 133 µm showed an increase from day 14 to day 28. All the cream samples thermosonicated at 152 µm showed a decrease in total plasmin activity from day 0 to day 28 except for the one treated for 180 s.

The effects of various processing techniques on plasmin activity have been reported. Experiments on inactivation of plasmin in simulated milk ultrafiltrate using pulsed electric field showed that approximately 60% of enzyme inactivation was achieved after 50 pulses with field strengths of 60 or 45 kV/cm at 10°C (Vega-Mercado et al., 1995). The inactivation effect increased to 90% at a temperature of 15°C. Pressurization at 400 MPa can decrease the total plasmin activity by approximately 20 to 30% in skim milk (García-Risco et al., 2003). When compared to the above inactivation techniques, ultrasound combined with heat was able to inactivate plasmin more considerably. Further, data on the regeneration of inactivated plasmin with respect to storage time is scarce and thus the findings of our studies help to throw light on the basic understanding of regeneration of plasmin activity after thermosonication.

3.3. Odor Evaluation

3.3.1. Preliminary Study

The observations of the two expert panelists who did odor analysis of the commercial pasteurized skim, reduced-fat and whole milk samples that were thermosonicated at 133 µm for 2.5 min are tabulated in Table 14. Samples were served in triplicate. Pasteurized skim and reduced-fat milk samples that were thermosonicated at 133 µm for 2.5 min had definite

intensities of offensive plastic/burnt/rubbery aroma, while the thermosonicated whole milk sample had pronounced intensity of the same. In addition, the intensity of cooked aroma was pronounced in reduced-fat and whole milk samples, whereas that of the skim milk sample was only slight. Of the three milk types, only the pasteurized and thermosonicated skim milk was acceptable in terms of odor profile.

Table 14. Effect of thermosonication on sensory properties of pasteurized milk samples (n=2; expert dairy judging panelists)

Pasteurized Milk: thermosonicated at 133µm for 2.5min	Intensity of Odor Attribute (defective)						Consumer Acceptability (odor profile)
	Plastic /burnt /rubbery			Cooked (custard)			
	S	D	P	S	D	P	
Skim		X		X			Yes
Reduced-fat		X				X	No
Whole			X			X	No

S – slight intensity; D – definite intensity; P – pronounced intensity

This rise of offensive odors in thermosonicated samples could be because of the chemical reactions taking place in the regions of high temperature and pressure, such as the interface of collapsing bubbles and surrounding liquid phase, and the formation of free radicals by pyrolysis (Henglein, 1995; Makino et al., 1983; Riesz and Kondo, 1992). Free radicals such as H• and OH• can induce auto-oxidation of lipids in milk resulting in the formation of aldehydes such as pentanal, hexanal and heptanal (Karatapanis et al., 2006; Toma et al., 2001). Because hexanal is a compound known to cause pungent, green and grassy off-flavor in sonicated sunflower oil (Chemat et al., 2004), sonication induced lipid oxidation could be a likely mechanism for the rise of compounds that might be responsible for the undesirable odor in milk during sonication.

Researchers have reported that sonicating milk of 1.5% fat content at 400 W power, 24 kHz frequency can cause undesirable rubbery odor (Riener et al., 2009). The concentration of

volatile alkenes such as 1-hexene, 1-octene, 1-nonene, 5-methyl-1,3-cyclopenatadiene, benzene, toluene, p-xylene, n-hexanal, and n-heptanal increased for the first 5 min of sonication. The authors stated that the nature of the chemical reactions that cause the undesirable odors cannot be associated with one or two of the components because milk is a complex matrix. Further, their experiments with Gas Chromatography – Olfactometry showed that none of the volatile compounds that arise or increase during sonication seem to have a significant individual contribution to the rubbery odor of the sonicated milk samples because of their weak and ill-defined aromas. However, a subjective descriptive analysis of odor attributes, which could explain whether ultrasound induces any detectable, undesirable odor in milk, was not performed. Thus, we decided to conducted descriptive analysis of thermosonicated skim milk and cream to test the hypothesis that the rise of rubber aroma is typical to the presence of fat in the substance. Also, we decided to perform thermosonication treatments directly on fresh raw skim milk and cream, with the assumption that thermosonication of commercial pasteurized sample is more likely to cause additive qualitative damage to milk than processing of raw milk. The results of our studies have shown that the extreme thermosonication treatment conditions that we used in our experiments do not significantly increase the undesirable rubbery and eggy odor attributes in both fresh skim milk and cream.

3.3.2. Descriptive Analysis

The triangle test conducted at the end of training session to determine the odor detection ability of the panelists showed that seven out of the nine panelists were able to correctly identify the odd sample in the trio, however, the panelists who were not able to correctly identify the odd sample were not removed from the panel. During the training sessions, the panelists agreed that nutty and creamy attributes are considered desirable attributes, whereas eggy and rubbery are considered offensives attributes that may result from milk processing. During the training and mock evaluation sessions, some of the panelists indicated that they perceived rubbery aroma only during the first sniff which seemed to disappear in the consecutive sniffs. A few indicated that they did not perceive rubbery aroma during the mock evaluation session but were able to perceive the rubbery aroma in the same samples during

the discussion session that followed the mock evaluation. In order to bring the panelists to a consensus, the panelists were trained for at least 10 h and were evaluated by two mock sessions before starting the actual evaluation session. At the end of training process the consensus among the panelists was evaluated using two mock evaluations. The first mock evaluation showed that 3 out of 9 panelists were not in consensus with the other panelists in scoring eggy odor attribute, 2 out of 9 panelists were not in consensus with the others in scoring nutty and rubbery odor attributes and 4 out of 9 was not in consensus with the others in scoring the creamy odor attribute. The second mock evaluation showed that 1 out of 9 panelists was not in consensus with the other panelists in scoring eggy and nutty odor attributes; 2 out of 9 panelists were not in consensus with the others in scoring rubbery odor attributes. All the panelists were in agreement with regards to the creamy attribute.

The mean intensity scores of the four odor attributes of skim milk samples evaluated by the trained panelists are shown in Figure 4. Analysis of variance revealed that the average nutty intensity of thermosonicated skim milk was significantly higher than the control pasteurized skim milk on week 0 ($p<0.01$) and week 1 ($p<0.05$). Also, average creamy intensity of thermosonicated skim milk was significantly higher than the control pasteurized skim milk on week 1 ($p<0.01$) and weeks 2 and 3 ($p<0.05$). The average intensities of offensive eggy and rubbery attributes were higher for the thermosonicated skim milk when compared to that of control, however, no significant differences were found. One of the reasons could be because of disagreement among panelists although the deviations from the average values for eggy attributes were similar to or less than that of nutty. It could also be seen that the standard deviation of the intensity of rubbery odor was relatively higher than that of the other odor attributes. This could be either because of the masking of the rubbery odor by the other odor attributes or because of disagreement among panelists (at the end of second mock evaluation, 2 out of 9 panelists were not in agreement with the other panelists in scoring the rubbery attribute) or difference in detection threshold for the rubbery attribute among the panelists. Also, the offensive rubbery attribute started to fade away in the thermosonicated skim milk samples with storage time and was less than that of the control by days 21 and 28. These findings imply that the extreme treatment conditions of thermosonication used in our research experiments may not have induced undesirable aroma in fresh raw skim milk.

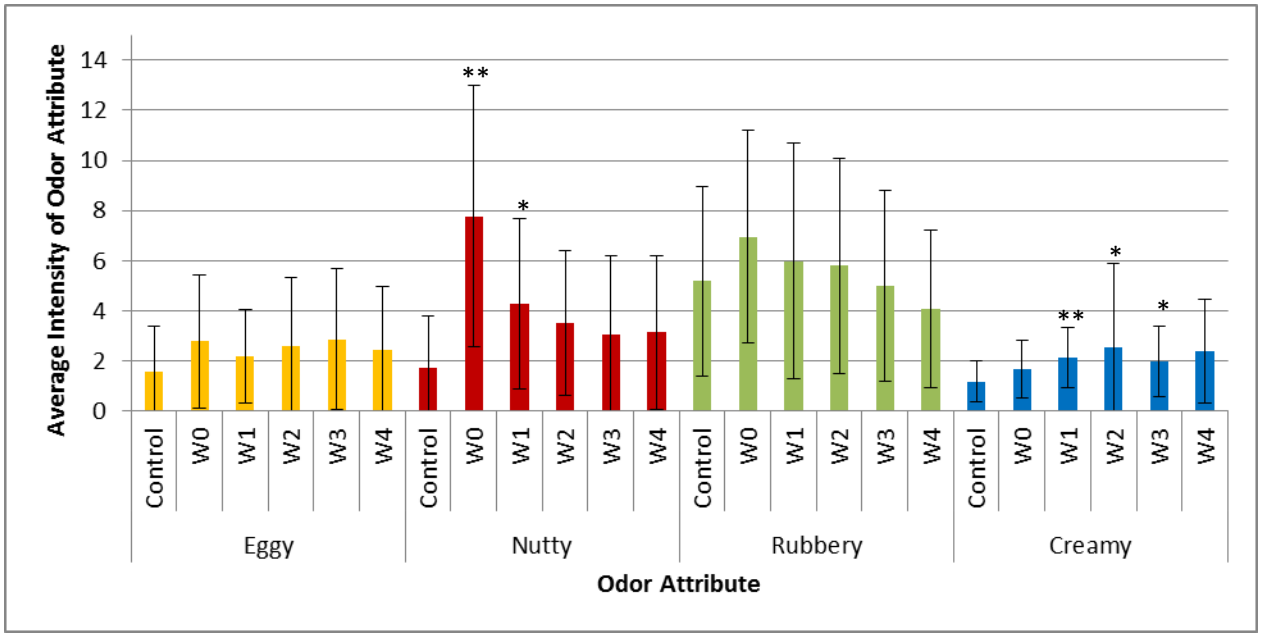

Figure 4. Intensity of odor attributes of skim milk thermosonicated at 152 µm for 3 min

*Significantly different from control: * ($p<0.05$); ** ($p<0.01$)*

The mean intensity scores of the four odor attributes of cream samples evaluated by the trained panelists are shown in Figure 5. The average eggy intensity of thermosonicated cream was less than that of control and decreased with storage time. The average nutty intensity of thermosonicated cream was higher than that of the control and it gradually increased from week 0 to week 2 and then gradually dropped on week 3 and 4. Similarly, the average rubbery intensity of thermosonicated cream was higher than that of the control and saw a gradual increase up to week 2. The average creamy intensity of thermosonicated cream decreased on week 1 and increased on week 2. However, ANOVA revealed no significant differences for any of the odor attributes between the thermosonicated cream and the control. These findings imply that the thermosonication treatment conditions used in our experiments may not have induced any significant undesirable changes in the odor attributes of cream.

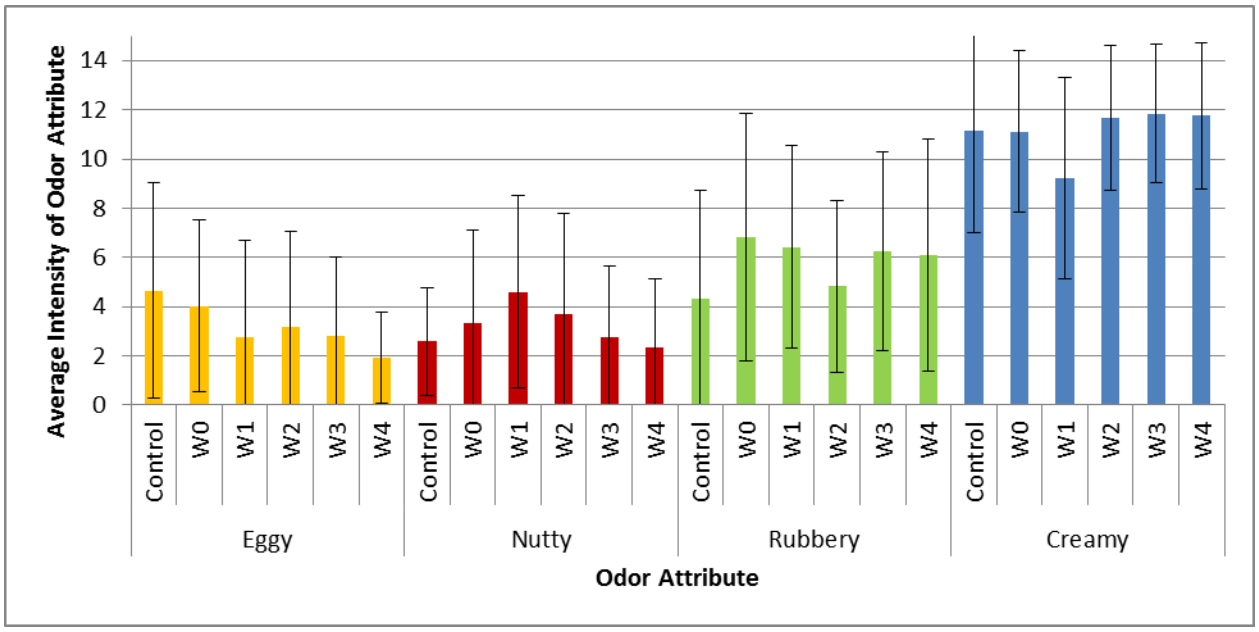

Figure 5. Intensity of odor attributes of cream thermosonicated at 152 µm for 3 min

No significant differences (p>0.05)

3.4. Lightness value of skim milk and cream

The L* value of thermosonicated skim milk and cream from experiment 1 are listed in Table 15 and that from experiment 2 are listed in Tables 16. The L* values range from 0 to 100; a higher L* value represents whiteness of the sample and a lower value represents black. The L* values of all thermosonicated skim milk samples (Tables 13 and 15) are higher than raw and heated skim milk samples implying that ultrasound had a homogenizing effect on the milk samples. We can also see that the L* values decreased gradually for most samples upon storage time. Decrease in L* value of milk can indicate the dissociation of casein micelles, because the micelles are responsible for scattering of the light (O'Sullivan et al., 2002) and L* is a measure of reflectance. Ultrasonic treatment at 35 kHz frequency (200 W) for 6 h is capable of disrupting re-assembled casein micelles (without breaking the peptide bond of casein chains) in casein solutions with the magnitude of disruption increasing with increasing output power (Madadlou et al., 2009). The decrease in lightness values of thermosonicated skim milk samples upon storage could be associated with the hydrolysis of casein micelles either because of regeneration plasmin activity or presence of other

Table 15. Lightness values of thermosonicated cream (Experiment I)

Treatment	L* value (mean ± SD) of skim milk			L* value (mean ± SD) of cream		
	Day 0	Day 14	Day28	Day 0	Day 14	Day 28
Raw	66.14 ± 1.10 [a]	--	--	74.94 ± 0.82 [a b]	--	--
TS at 107µm for 1 min	67.95 ± 1.47 [a]	67.74 ± 1.37 [a]	67.09 ± 3.09 [a]	77.46 ± 2.11 [a]	75.65 ± 0.11 [a b]	74.35 ± 1.64 [a b]
TS at 107µm for 2 min	68.02 ± 1.51 [a]	67.74 ± 1.47 [a]	66.77 ± 0.28 [a]	76.26 ± 0.39 [a]	75.40 ± 0.40 [a b]	70.84 ± 0.83 [b]
TS at 107µm for 2.5 min	69.24 ± 2.50 [a]	66.82 ± 2.63 [a]	67.32 ± 2.39 [a]	77.73 ± 1.71 [a]	74.83 ± 0.39 [a b]	76.82 ± 3.78 [a]
TS at 107µm for 3 min	69.29 ± 2.69 [a]	65.72 ± 1.93 [a]	65.76 ± 0.23 [a]	77.41 ± 0.71 [a]	75.68 ± 0.48 [a b]	74.61 ± 2.18 [a b]
TS at 133µm for 1 min	70.58 ± 0.70 [a]	65.76 ± 1.94 [a]	68.79 ± 0.35 [a]	75.36 ± 0.48 [a b]	74.93 ± 0.53 [a b]	73.98 ± 0.74 [a b]
TS at 133µm for 2 min	71.36 ± 1.24 [a]	66.84 ± 0.15 [a]	67.85 ± 0.23 [a]	74.97 ± 0.13 [a b]	75.57 ± 0.32 [a b]	76.73 ± 0.76 [a]
TS at 133µm for 2.5 min	69.72 ± 0.76 [a]	66.88 ± 0.17 [a]	67.86 ± 1.19 [a]	75.22 ± 0.47 [a b]	75.50 ± 0.97 [a b]	75.89 ± 0.69 [a b]
TS at 133µm for 3 min	71.40 ± 1.24 [a]	67.14 ± 0.04 [a]	68.94 ± 0.38 [a]	75.95 ± 0.58 [a]	76.01 ± 0.28 [a]	75.05 ± 2.77 [a b]
TS at 152 µm for 1 min	68.90 ± 0.03 [a]	68.04 ± 2.79 [a]	68.09 ± 0.72 [a]	75.64 ± 1.74 [a b]	75.58 ± 0.19 [a b]	76.99 ± 0.15 [a]
TS at 152 µm for 2 min	68.17 ± 0.79 [a]	68.15 ± 2.84 [a]	65.31 ± 2.15 [a]	76.34 ± 0.78 [a]	75.96 ± 0.12 [a]	74.98 ± 2.60 [a b]
TS at 152 µm for 2.5 min	71.19 ± 2.76 [a]	67.58 ± 4.46 [a]	69.45 ± 1.39 [a]	75.41 ± 0.45 [a b]	75.67 ± 0.59 [a b]	75.44 ± 0.69 [a b]
TS at 152 µm for 3 min	69.34 ± 0.95 [a]	67.57 ± 4.51 [a]	68.25 ± 0.69 [a]	77.79 ± 1.40 [a]	76.93 ± 0.06 [a]	73.89 ± 0.61 [a b]

TS - thermosonicated; Means with same letter within skim milk/cream columns are not significantly different

Table 16. Lightness values of thermosonicated skim milk and cream (Experiment II)

Treatment given to skim milk sample	L* value (mean ± SD) of Skim milk			L* value (mean ± SD) of Cream		
	D1	D15	D30	D1	D15	D30
Raw	77.36 ± 0.65 ^{abcd}	--	--	82.81 ± 0.40 ^{abcd}	--	--
Heated to 72°C for 1 min	77.29 ± 0.15 ^{abcd}	78.23 ± 0.18 ^{abc}	75.78 ± 0.55 ^{d}	84.93 ± 0.56 ^{abc}	79.28 ± 3.58 ^{d}	79.63 ± 0.69 ^{cd}
Heated to 72°C for 3 min	77.85 ± 0.87 ^{abcd}	77.11 ± 0.94 ^{abcd}	76.23 ± 0.07 ^{cd}	84.40 ± 2.92 ^{abcd}	81.29 ± 0.33 ^{abcd}	79.19 ± 1.02 ^{d}
TS at 133 µm for 1 min	77.52 ± 0.67 ^{abcd}	78.42 ± 0.90 ^{ab}	76.76 ± 0.21 ^{bcd}	86.44 ± 1.12 ^{a}	83.67 ± 0.28 ^{abcd}	80.59 ± 0.86 ^{bcd}
TS at 133 µm for 3 min	78.57 ± 0.09 ^{ab}	79.06 ± 0.03 ^{a}	76.86 ± 0.04 ^{bcd}	86.48 ± 1.17 ^{a}	83.60 ± 0.47 ^{abcd}	80.23 ± 0.98 ^{cd}
TS at 152 µm for 1 min	77.37 ± 0.18 ^{abcd}	78.35 ± 0.46 ^{ab}	76.71 ± 0.26 ^{bcd}	85.63 ± 0.47 ^{ab}	82.86 ± 1.17 ^{abcd}	80.13 ± 0.94 ^{cd}
TS at 152 µm for 3 min	78.66 ± 0.91 ^{ab}	77.21 ± 0.30 ^{abcd}	76.95 ± 0.34 ^{bcd}	85.72 ± 1.29 ^{ab}	84.35 ± 0.33 ^{abcd}	82.31 ± 0.04 ^{abcd}

TS - thermosonicated; Means with same letter within skim milk/cream columns are not significantly different

proteases. Casein hydrolysis of plasmin reduces the negative charges on the micelles (Crudden et al., 2005) and the resulting inter-micellar repulsion affects light scattering properties, thus leading to reduced lightness value. However, the decrease in lightness values after thermosonication (at any treatment) was not significant implying that the thermosonication treatment conditions used in our experiments would not adversely affect the appearance of skim milk. Further, the L* values of the thermosonicated skim milk samples on day 0, 14 and 28 (Table 15) were not significantly different ($p>0.05$) from that of the untreated skim milk (measured on day 0). Similar results were observed in experiment 2 (Table 16) where the L* values of the thermosonicated skim milk samples on day 0, 15 and 30 were not significantly different ($p>0.05$) from that of the untreated skim milk (measured on day 0).

Similar to the results of experiment 1, lightness values of thermosonicated skim milk and cream samples were higher than that of raw samples in experiment 2. Lightness values of

cream samples on day 30 is less than that of day 1 which could be because of fat aggregation in cream samples. A decrease in the lightness value of most cream samples from day 0 to day 28 was observed in experiment 1 (Tables 15) and from day 1 to day 30 in experiment 2 (Table 16). This decrease could be attributed to the formation of fat aggregates over time resulting in reduced reflectance and thus lesser L* values. However, lightness values of treated cream samples on day 0, 14, and 28 in experiment 1 (Table 15) and on day 1, 15 and 30 in experiment 2 (Table 16) were not significantly different from that of untreated cream sample (measured on day 0) implying that the thermosonication treatment conditions used in our experiments would not adversely affect the appearance of cream.

3.5. **Viscosity of Milk and Cream**

The average viscosity values of pasteurized skim, reduced-fat and whole milk samples that were thermosonicated at 133 µm for 2.5 min are shown in Table 17.

Table 17. Viscosity of pasteurized thermosonicated milk samples (Preliminary Studies)

Sample	Viscosity (mean ± SD), Ns/m^2
Skim milk control	$(1.09 \pm 0.01) \times 10^{-3\ a}$
Skim milk TS	$(1.02 \pm 0.10) \times 10^{-3\ a}$
Reduced-fat milk control	$(1.25 \pm 0.02) \times 10^{-3\ a}$
Reduced-fat milk TS	$(1.32 \pm 0.13) \times 10^{-3\ a}$
Whole control milk	$(1.51 \pm 0.26) \times 10^{-3\ a}$
Whole milk TS	$(1.33 \pm 0.09) \times 10^{-3\ a}$

TS - Thermosonication treatment at 133 µm, 2.5 min; Means with same letter between skim, reduced-fat or whole milk columns are not significantly different

Thermosonication at 133 µm, 2.5 min did not affect the viscosity of pasteurized skim, reduced-fat and whole milk (Table 17). Researchers have reported that prolonged ultrasound treatment is capable of denaturing milk proteins. Cavitation caused by sonication can denature the proteins i.e. loss of tertiary structure or unfolding of globular proteins which in

turn swells up resulting in increased hydrodynamic radius and greater molecular inter-entanglements. All of these lead to increase in viscosity (Ipsen et al., 2000).

Viscosity values of raw, heated and thermosonicated skim milk and cream samples measured on D6, D15 and D30 are listed in Tables 18. No significant differences were observed in the viscosity values of skim milk samples because of heating and thermosonication over the 30-day period. However, viscosity of heated cream samples was significantly higher than that of raw cream samples whereas no significant differences were observed between raw and thermosonicated cream samples. Thus no adverse effects on viscosity were observed in both skim milk and cream samples because of thermosonication over a 30-day period.

Table 18. Viscosity of thermosonicated skim milk samples

Treatment	Viscosity of skim milk, (mean±SD) x 10^{-3}, Pa.s			Viscosity of cream, (mean±SD) x 10^{-3}, Pa.s		
	D1	D15	D30	D1	D15	D30
Raw	1.31 ± 0.30 [a]	--	--	2.72 ± 0.07 [b]	--	--
Heated to 72°C for 1 min	1.41 ± 0.14 [a]	1.23 ± 0.04 [a]	1.76 ± 0.35 [a]	17.79 ± 0.46 [a]	14.48 ± 2.99 [a]	16.71 ± 5.92 [a]
Heated to 72°C for 3 min	1.42 ± 0.14 [a]	1.25 ± 0.03 [a]	1.93 ± 0.44 [a]	10.02 ± 1.52 [a]	3.46 ± 0.51 [b]	8.85 ± 5.43 [b]
TS at 133 µm for 1 min	1.45 ± 0.09 [a]	1.68 ± 0.23 [a]	1.68 ± 0.11 [a]	4.05 ± 0.19 [b]	4.01 ± 0.27 [b]	3.08 ± 0.38 [b]
TS at 133 µm for 3 min	1.60 ± 0.05 [a]	1.91 ± 0.03 [a]	1.70 ± 0.08 [a]	3.26 ± 0.33 [b]	3.04 ± 0.06 [b]	3.21 ± 0.12 [b]
TS at 152 µm for 1 min	1.55 ± 0.38 [a]	1.86 ± 0.74 [a]	1.62 ± 0.09 [a]	2.84 ± 0.41 [b]	2.81 ± 0.11 [b]	4.13 ± 0.37 [b]
TS at 152 µm for 3 min	1.39 ± 0.00 [a]	1.60 ± 0.13 [a]	1.62 ± 0.12 [a]	4.46 ± 0.75 [b]	4.22 ± 0.30 [b]	4.23 ± 0.11 [b]

TS - Thermosonicated; Means with same letter within skim milk/cream columns are not significantly different

3.6. Milk Fat Globule Size

The sauter diameter, volume-weighted diameter of fat globules in raw, heated, and thermosonicated skim milk and cream samples measured on D6, D15 and D30 are listed

Tables 18 and 19, respectively. The specific area [$S = 6/(\rho * D_{3,2})$], of fat globules in raw, heated and thermosonicated skim milk and cream samples are listed in Table 20. Thermosonication caused significant decreased in the sauter diameter ($D_{3,2}$) and volume-weighted diameter ($D_{3,4}$) and a significant increase in specific area of fat globules in both skim milk and cream when compared to the raw and heated samples (Tables 19, 20 and 21). No differences in $D_{3,2}$, $D_{4,3}$ and specific area values were observed between raw and heated skim milk samples over the 30-day period. This implies that ultrasound has caused homogenization of skim milk. It can also be observed that longer sonication time resulted in smaller fat globules i.e. better homogenization effect. Our results agree with that of Wu et al. (2001) who reported that longer sonication time periods improved homogenization effect in milk. No significant differences were observed among the $D_{3,2,}$ $D_{4,3}$ and specific area values of both thermosonicated skim milk and cream samples over the 30-day storage period.

Table 19. Fat globule size in skim milk (Experiment II)

Sample	Sauter diameter (mean ± SD), $D_{3,2}$ (µm)			Volume-weighted diameter (mean ± SD), $D_{4,3}$ (µm)		
	D6	D15	D30	D6	D15	D30
Raw	2.65 ± 0.07 ^{a}	--	--	3.12 ± 0.17 ^{d}	--	--
Heated to 72°C for 1 min	2.63 ± 0.04 ^{a}	2.59 ± 0.00 ^{a}	2.51 ± 0.11 ^{a}	3.13 ± 0.06 ^{b}	3.06 ± 0.01 ^{e}	2.97 ± 0.13 ^{c}
Heated to 72°C for 3 min	2.56 ± 0.04 ^{a}	2.57 ± 0.04 ^{a}	2.57 ± 0.00 ^{a}	3.00 ± 0.12 ^{a}	2.99 ± 0.10 ^{f}	3.04 ± 0.00 ^{e}
TS at 133 µm for 1 min	0.40 ± 0.11 ^{bc}	0.32 ± 0.00 ^{bcd}	0.33 ± 0.00 ^{bcd}	0.93 ± 0.50 ^{g}	0.60 ± 0.01 ^{j}	1.21 ± 0.08 ^{j}
TS at 133 µm for 3 min	0.20 ± 0.00 ^{d}	0.21 ± 0.01 ^{cd}	0.23 ± 0.00 ^{cd}	0.21 ± 0.00 ^{ih}	0.54 ± 0.40 ^{k}	0.65 ± 0.31 ^{k}
TS at 152 µm for 1 min	0.48 ± 0.03 ^{b}	0.26 ± 0.03 ^{cd}	0.47 ± 0.00 ^{b}	1.25 ± 0.08 ^{i}	0.77 ± 0.51 ^{k}	1.26 ± 0.02 ^{k}
TS at 152 µm for 3 min	0.22 ± 0.03 ^{cd}	0.20 ± 0.00 ^{d}	0.25 ± 0.00 ^{cd}	0.30 ± 0.11 ^{h}	0.22 ± 0.00 ^{k}	0.92 ± 0.12 ^{k}

TS - Thermosonicated; Means with same letter within skim milk/cream columns are not significantly different

Table 20. Fat globule size in cream (Experiment II)

Sample	$D_{3,2}$ (mean ± SD), µm			$D_{4,3}$ (mean ± SD), µm		
	D6	D15	D30	D6	D15	D30
Raw	3.62 ± 0.03 [a]	--	--	4.10 ± 0.01 [de]	--	--
Heated to 72°C for 1 min	3.81 ± 0.01 [a]	3.81 ± 0.00 [a]	4.24 ± 0.05 [a]	4.54 ± 0.07 [cd]	4.59 ± 0.06 [cd]	6.61 ± 0.38 [a]
Heated to 72°C for 3 min	4.02 ± 0.01 [a]	3.24 ± 0.00 [a]	3.69 ± 0.02 [a]	4.85 ± 0.07 [c]	3.67 ± 0.00 [ef]	5.72 ± 0.07 [b]
TS at 133 µm for 1 min	0.70 ± 0.06 [bc]	0.73 ± 0.01 [bc]	0.72 ± 0.01 [b]	1.87 ± 0.03 [g]	3.55 ± 0.01 [f]	3.74 ± 0.40 [ef]
TS at 133 µm for 3 min	0.62 ± 0.01 [c]	0.54 ± 0.05 [bc]	0.56 ± 0.00 [bc]	1.20 ± 0.00 [h]	1.05 ± 0.06 [h]	1.13 ± 0.01 [h]
TS at 152 µm for 1 min	0.60 ± 0.03 [b]	0.57 ± 0.00 [bc]	0.60 ± 0.02 [b]	1.14 ± 0.00 [h]	1.21 ± 0.00 [h]	2.04 ± 0.07 [g]
TS at 152 µm for 3 min	0.72 ± 0.02 [c]	0.57 ± 0.03 [c]	0.59 ± 0.01 [bc]	1.28 ± 0.02 [h]	1.15 ± 0.04 [h]	1.23 ± 0.01 [h]

TS - Thermosonicated; Means with same letter within skim milk/cream columns are not significantly different

Table 21. Specific area of fat globules of skim milk and cream

Treatment	Specific area (mean ± SD) of skim milk, $\mu m^2 g^{-1}$			Specific area (mean ± SD) of cream, $\mu m^2 g^{-1}$		
	D6	D15	D30	D6	D15	D30
Raw	2.46 ± 0.07 [f]	--	--	1.80 ± 0.02 [f]	--	--
Heated to 72°C for 1 min	2.48 ± 0.04 [f]	2.52 ± 0.00 [f]	2.60 ± 0.11 [f]	1.71 ± 0.00 [f]	1.71 ± 0.00 [f]	1.54 ± 0.02 [f]
Heated to 72°C for 3 min	2.55 ± 0.04 [f]	2.54 ± 0.04 [f]	2.54 ± 0.00 [f]	1.62 ± 0.00 [f]	2.01 ± 0.00 [f]	1.77 ± 0.01 [f]
TS at 133 µm for 1 min	16.62 ± 5.03 [de]	20.61 ± 0.14 [cd]	19.70 ± 0.00 [cde]	9.31 ± 0.78 [cde]	8.92 ± 0.13 [e]	9.01 ± 0.06 [e]
TS at 133 µm for 3 min	33.36 ± 0.12 [a]	30.69 ± 1.12 [ab]	28.86 ± 0.18 [ab]	10.52 ± 0.22 [bdc]	12.03 ± 1.04 [a]	11.71 ± 0.09 [ab]
TS at 152 µm for 1 min	13.63 ± 0.79 [e]	25.63 ± 3.23 [bc]	13.98 ± 0.02 [de]	10.80 ± 0.56 [abc]	11.43 ± 0.01 [ab]	10.94 ± 0.38 [ab]
TS at 152 µm for 3 min	29.58 ± 4.31 [ab]	33.11 ± 0.24 [a]	26.09 ± 0.00 [bc]	9.11 ± 0.22 [de]	11.41 ± 0.55 [ab]	11.11 ± 0.16 [ab]

TS - Thermosonicated; Means with same letter within skim milk/cream columns are not significantly different

3.7. Microbiological Analysis

Total aerobic bacteria count and coliform count of raw whole milk (collected for Experiment II) is listed in Table 22. Coliform counts indicate the presence of fecal bacteria in fluid milk and good quality raw milk contains approximately <100 CFU/mL coliforms (Reinemann et al. 1997). The high coliform count in the raw milk in the present study could be due to unhygienic milk collection practices by the staff person.

Table 22. Microbial analysis of raw whole milk (Experiment II)

Analyses	Log (mean±SD), CFU/mL
Total aerobic bacteria	3.92±3.30
Coliforms	3.65±3.05

Coliform count and Total aerobic bacteria count of raw, heated and thermosonicated skim milk and cream samples measured at three times (on days 1 (D1), 15 (D15) and 30 (D30)) (experiment II) are listed in Tables 23 and 24, respectively. Coliforms were eliminated from milk by all heat and thermosonication treatments; no coliforms grew on plates poured on D1, D15 or D30. All heating and thermosonication treatments caused more than 1.86 log reduction of total aerobic bacteria both in skim milk and cream treatments (D1). The dilution range (10^{-2}) selected (based upon prediction of counts) to determine the total aerobic bacteria counts in treated skim milk and cream samples did not enable us to enumerate less than 100 CFU/mL (i.e. 2.00 log CFU/mL).

After two weeks of refrigerated storage (D15), total bacterial count of skim milk heated for 1 min was still less than 2 log CFU/mL whereas an increase in total aerobic bacteria count was observed in skim milk samples heated for 3 min (more than 0.72 log increase) and skim milk samples thermosonicated at 152 µm for 1 min (more than 0.37 log increase) and 3 min (more than 0.31 log increase). Similarly, an increase in total aerobic bacteria was observed in all thermosonicated cream samples on D15, however an increase in total aerobic bacteria was not observed in heated cream samples on D15. The highest increase was observed in cream thermosonicated at 133 µm for 3 min (more than 3.35 log increase on D15). However on

D30, the total bacterial numbers decreased for both skim milk and cream samples (heated and thermosonicated). This could be explained by the fact that the bacteria in the treated samples might have been in the logarithmic growth phase when measured on D15 and reached their death phase on D30. Further, it is possible that the skim milk sample thermosonicated at 133 µm for 3 min could have exceeded 20,000 CFU/mL but the dilutions selected were unable to reveal the true counts.

Conventional pasteurization conditions have been selected to effectively destroy all pathogens (5 log) from milk but some spoilage microorganisms survive and proliferate. In the present study, thermosonication (133 µm for 1 min and 152 µm for 1 and 3 min) was equally effective to heat treatment (72°C for 1 and 3 min) for eliminating coliforms. In skim milk, thermosonication was more effective in reducing microbial numbers than heating, whereas in cream samples heating was more effective than thermosonication in reducing total aerobic bacteria in cream. This is likely because of the protection offered by fat to the bacteria in the cream samples. It is established that dairy products with fat content above 10% (or added sugar) must be pasteurized to 66°C for 30 min or 75°C for 16 s (FDA, 2009) and ice cream mix or egg nog must be pasteurized at least 69°C for 30 min or 80°C for not less than 25 s (FDA, 2009) because fat and sugar have a protective effect over the microbes.

On days 1, 15 and 30, the total aerobic bacteria count and coliform count of all heated and all thermosonicated skim milk (except TS at 133 µm for 3 min) and cream samples were within quality standards established by the USDA HHS (FDA, 2009) for “Grade A” pasteurized milk (less than 20,000 CFU/mL and less than 10 CFU/mL, respectively), implying that the samples had not reached the end of their microbiological shelf-life by D30. These results suggest that ultrasound may serve as an adjunct to thermal pasteurization to increase the shelf-life of fluid milk by not only decreasing the activity of proteases but also by inactivating high numbers of spoilage microorganisms.

Table 23. Coliform count in skim milk and cream samples (Experiment II)

Sample	Coliform count of skim milk, log (mean±SD), CFU/mL			Coliform count of cream, log (mean±SD), CFU/mL		
	D1±24 h	D15	D30	D1±24 h	D15	D30
Raw	3.53±2.45 E	--	--	>3.40E	--	--
Heated to 72°C for 1 min	<10E	<10E	<10E	<10E	<10E	<10E
Heated to 72°C for 3 min	<10E	<10E	<10E	<10E	<10E	<10E
TS at 133 µm for 1 min	<10E	<10E	<10E	<10E	<10E	<10E
TS at 133 µm for 3 min	<10E	<10E	<10E	<10E	<10E	<10E
TS at 152 µm for 1 min	<10E	<10E	<10E	<10E	<10E	<10E
TS at 152 µm for 3 min	<10E	<10E	<10E	<10E	<10E	<10E

TS = thermosonicated; E = estimated (because of low counts on plates)

Table 24. Total aerobic bacteria count of skim milk and cream (Experiment II)

Sample	Total aerobic bacteria count, skim milk, log (mean±SD), CFU/mL			Total aerobic bacteria count, cream, log (mean±SD), CFU/mL		
	D1±24 h	D15	D30	D1±24 h	D15	D30
Raw	3.86±2.33E	--	--	3.97±3.31	--	--
Heated to 72°C for 1 min	<2.00E	<2.00E	<2.00E	<2.00E	<2.00E	<2.00E
Heated to 72°C for 3 min	<2.00E	2.72±3.06	<2.00E	<2.00E	<2.00E	<2.00E
TS at 133 µm for 1 min	<2.00E	U	<2.00E	<2.00E	2.54±7.85	<3.00E
TS at 133 µm for 3 min	<2.00E	<2.00E	>3.40E	<2.00E	3.35±2.80	<2.00E
TS at 152 µm for 1 min	<2.00E	2.37±1.70	<3.00E	<2.00E	2.38±1.85	<2.00E
TS at 152 µm for 3 min	<2.00E	2.31±1.89	<3.00E	<2.00E	2.43±1.15	<2.00E

TS = thermosonicated; U = uncountable because of spreaders; E = estimated (because of low counts on plates)

CHAPTER 4. SUMMARY AND CONCLUSIONS

Our results showed that ultrasound, when combined with heat, is capable of decreasing the activity of *Staphylococcus aureus* protease in skim (by approx. 72%), reduced-fat (by approx. 92%) and whole milk (by approx. 92%) and total plasmin activity in fresh skim milk (by approx. 81 to 94%) and cream (by approx. 96%). The total plasmin activity increased in skim milk over a storage period of four weeks, whereas it did not increase in cream over the same storage period. Results of descriptive analysis showed that the extreme treatment conditions of thermosonication used in our research experiments did not significantly increase the intensity of offensive rubbery and eggy odor attributes in skim milk and cream. Rheological experiments showed that thermosonication did not cause any changes in viscosity of commercial pasteurized skim, reduced-fat and whole milk when measured on day 1 or any adverse changes in raw skim milk and cream over a 30-day period. Thermosonication did not adversely affect the fat, solids-non-fat and protein content of skim milk. Thermosonication did not cause significant differences in the lightness (L*) value of skim milk and cream and thus would not adversely affect the appearance of skim milk and cream. Thermosonication caused a significant reduction in fat globule size in both skim milk and cream and thus had a homogenizing effect. Microbial analysis showed that thermosonication completely destroyed all coliforms and caused more than a 99% reduction in total bacterial count in both skim milk and cream. Total aerobic bacteria count of skim milk and cream on day 30 were still within the limits established by FDA for “Grade A” pasteurized milk. The outcome of this research has laid the groundwork for future work to establish the credibility of ultrasound in commercial dairy processing to extend milk shelf-life.

CHAPTER 5. RECOMMENDATIONS FOR FUTURE WORK

The food and dairy industries have a variety of uses for ultrasound, and thermosonication may serve to extend the shelf-life of fluid milk. Although ultrasound has a lot of potential applications in processing of fluid milk and other dairy products, further research needs to done to prove its reliability upon scaling from lab to the processing facility. An extended storage study investigating the effect of different treatment combinations of ultrasound and heat on other proteases such as cathepsins, proteases from different microbial sources and other enzymes such as lipases must be performed. Future research should also be conducted to determine shelf-life of thermosonicated milk (at different treatment conditions) in relation to microbial spoilage, because microorganisms are not entirely eliminated by the thermosonication treatment conditions used in our research. Because the growth of microorganisms can cause not only protein gelation but also off-flavors in milk, thermosonication treatment conditions must be optimized to completely inactivate the microorganisms in milk. The technique also needs to be evaluated for its suitability upon scale-up, especially in terms of microbiological safety and quality and sensory properties (appearance, flavor and odor). Future work should also involve determination of how to install thermosonication setup in commercial dairy processing facilities. The cost involved in installation of the sonication setup in a continuous processing plant and the energy consumption should be comparative to the commercial thermal processing technique.

REFERENCES

1. Alichanidis, E., J. H. M. Wrathall, and A. T. Andrews. 1986. Heat stability of plasmin (milk proteinase) and plasminogen. J. Dairy Res. 53:259-269.
2. Allen, J. C., and G. Joseph. 1985. Review article: Deterioration of pasteurized milk on storage. J. Dairy Res. 52:469–486.
3. AOAC. 2005. Fat in raw milk: Babcock method (989.04). Official Methods of Analysis. 18th ed. Association of Official Analytical Chemists International. Gaithersburg, MD.
4. Bastian, E.D., K.G. Hansen, and R. J. Brown. 1993. Inhibition of plasmin by beta-lactoglobulin using casein and a synthetic substrate. J. Dairy Sci. 76: 3354-3361.
5. Bastian, E. D., and R. J. Brown. 1996. Plasmin in milk and dairy products: An update. Int. Dairy J. 6: 435-457.
6. Bendicho, S., G. Martí, T. Hernáandez, and O. Martín. 2002. Determination of proteolytic activity in different milk systems. Food Chem. 79: 245–249.
7. Bendicho, S., A. R. Marselles-Fontanet, G. V. Barbosa-Canovas, and O. Martin-Belloso. 2005. High intensity pulsed electric fields and heat treatments applied to a protease from *Bacillus subtilis*. A comparison study of multiple systems. J. Food Eng. 69: 317–323.
8. Bermúdez-Aguirre, D., M. G. Corradini, R. Mawson, and G. V. Barbosa-Cánovas. 2009. Modeling the inactivation of *Listeria innocua* in raw whole milk treated under thermo-sonication. Innov. Food Sci. Emerg. Technol. 10: 172-178.
9. Bilbao-Sainz, C., F. L. Younce, B. Rasco, and S. Clark. 2009. Protease stability in bovine milk under combined thermal-high hydrostatic pressure treatment. Innov. Food Sci. Emerg. Technol. 10: 314–320.
10. Boor, K. J., and D. N. Nakimbugwe. 1998. Quality and stability of 2% fat ultrapasteurized fluid milk products. Dairy Food Environ. Sanit. 18: 78–82.
11. Borda, D., Indrawati, C. Smout, A. Van Loey, and M. Hendrickx. 2004. High pressure thermal inactivation kinetics of a plasmin system. J. Dairy Sci. 87: 2351–2358.
12. Chapman, K. W., and K. J. Boor. 2001. Acceptance of 2% ultrapasteurized milk by consumers, 6–11 years old. J. Dairy Sci. 84: 951–954.

13. Chapman, K. W., H. T. Lawless, K. J., and Boor. 2001. Quantitative descriptive analysis and principal component analysis for sensory characterization of ultrapasteurized milk. J. Dairy Sci. 84: 12–20.
14. Chemat, F., I. Grondin, P. Costes, L. Moutoussamy, A. S. C. Sing, and J. Smadja. 2004. High power ultrasound effects on lipid oxidation of refined sunflower oil. Ultrason. Sonochem. 11: 281–285.
15. Chen, L., R. M. Daniel, and T. Coolbear. 2003. Detection and impact of protease and lipase activities in milk and milk powders. Int. Dairy J. 13: 255-275.
16. Christen, G. L., and R.T. Marshall. 1984. Selected properties of lipase and protease of *Pseudomonas fluorescens* 27 produced in four media. J. Dairy Sci. 67:1680-1687.
17. Christensen, K. R., and G. A. Reineccius. 1992. Gas chromatographic analysis of volatile sulfur compounds from heated milk using static headspace sampling. J. Dairy Sci. 75: 2098–2104.
18. Clare, D. A., W. S. Bang, G. Cartwright, M. A. Drake, P. Coronel, and J. Simunovic. 2005. Comparison of sensory, microbiological, and biochemical parameters of microwave versus indirect UHT fluid milk during storage. J. Dairy Sci. 88: 4172–4182.
19. Code of Federal Regulations. 2011. Food Labeling: Specific requirements for nutrient content claims (part 101.62). Code of Federal Regulations, Title 21, vol 2. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm. Accessed on 08/08/2011.
20. Code of Federal Regulations. 2011. Milk and Cream: Requirements for specific standardized milk and cream (part 131.150).
21. Code of Federal Regulations, Title 21, vol 2. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=131.150. Accessed on 08/08/2011.
22. Contarini, G., and M. Povolo. 2002. Volatile fraction of milk: Comparison between purge and trap and solid phase microextraction techniques. J. Agric. Food Chem. 50: 7350–7355.
23. Crudden, A., D. Afoufa-Bastien, P.F. Fox, G. Brisson, and A.L. Kelly. 2005. Effect of hydrolysis of casein by plasmin on the heat stability of milk. Int. Dairy J. 15: 1017–1025.

24. Daniel, R. M. 1996. The upper limits of enzyme thermal stability. Enzyme Microb. Tech. 19: 74-79.
25. Davies, K. J. A. 1987. Protein damage and degradation by oxygen radicals. I. General aspects. J. Biol. Chem. 262: 9895-9901.
26. Davies, K. J. A., and M. E. Delsignore. 1987. Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure. J. Biol. Chem. 262: 9908-9913.
27. Davies, K. J. A., M. E. Delsignore, and S. W. Lin. 1987. Protein damage and degradation by oxygen radicals. III. Modification of amino acids. J. Biol. Chem. 262: 9902-9907.
28. Dumay, E. M., M. T. Kalichevsky, and J. C. Cheftel. 1994. High-pressure unfolding and aggregation of β-lactoglobulin and the baroprotective effects of sucrose. J. Agric. Food Chem. 42: 1861-1868.
29. Earnshaw, R. G., J. Appleyard, and R. M. Hurst. 1995. Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. Int. J. Food Microbiol. 28: 197-219.
30. Elliott, A. J., N. Datta, B. Amenu, and H. C. Deeth. 2005. Heat-induced and other chemical changes in commercial UHT milks. J. Dairy Res. 72: 442–446.
31. ERS. 2003. Trends in U.S. Per Capita Consumption of Dairy Products, 1909 to 2001. Amber Waves. Available: http://www.ers.usda.gov/amberwaves/june03/datafeature/. Accessed on 07/05/2011.
32. FDA. 2009. Grade "A" Pasteurized Milk Ordinance. U.S. Dept. of Health and Human Services, Public Health Service. Available: http://www.fda.gov/downloads/Food/FoodSafety/Product-SpecificInformation/MilkSafety/NationalConferenceonInterstateMilkShipmentsNCIMSModelDocuments/UCM209789.pdf. Accessed on 07/05/2011.
33. Fellows, P. J. 2000. Food Processing Technology: Principles and Practice. 2nd ed. CRC Press, Boca Raton, FL.
34. Floros, J. D., and H. Liang. 1994. Acoustically assisted diffusion through membranes and biomaterials. Food Technol. 48: 79-84.

35. Floury, J., A. Desrumaux, and J. Lardieres. 2000. Effect of high pressure homogenization on droplet size distributions and rheological properties of model oil-in-water emulsions. Innov. Food Sci. Emerg. Technol. 1: 127–133.
36. Floury, J., N. Grosset, N. Leconte, M. Pasco, M. N. Madec, and R. Jeantet. 2006. Continuous raw skim milk processing by pulsed electric field at non-lethal temperature: effect on microbial inactivation and functional properties. Le Lait. 86: 43-57.
37. Fox, P. F., J. R. Whitaker, A. G. J. Voragen, and D. W. S. Wong. 2003. Significance of indigenous enzymes in milk and dairy products. Handbook of food enzymology. Marcel Dekker, New York.
38. Fox, P. F., and A. L. Kelly. 2006. Indigenous enzymes in milk: Overview and historical aspects - Part 1. Int. Dairy J. 16: 500-516.
39. Fox, P. F., and A. L. Kelly. 2006. Indigenous enzymes in milk: Overview and historical aspects - Part 2. Int. Dairy J. 16: 517-532.
40. Fromm, H. I. and K. J. Boor. 2004. Characterization of pasteurized fluid milk shelf-life attributes. J. Food Sci. 69: 207-214.
41. García, M. L., J. Burgos, B. Sanz, and J. A. Ordóñez. 1989. Effect of heat and ultrasonic waves on the survival of two strains of *Bacillus subtilis*. J. Appl. Bacteriol. 67: 619-628.
42. García-Risco, M. R., I. Recio, E. Molina, and R. López-Fandiño. 2003. Plasmin activity in pressurized milk. J. Dairy Sci. 86: 728-734.
43. Hayes, W., C. H. White, and M. A. Drak. 2002. Sensory aroma characteristics of milk spoilage by *Pseudomonas* species. J. Food Sci. 67:861–867.
44. Henglein, A. 1995. Chemical effects of continuous and pulsed ultrasound in aqueous solutions. Ultrason. Sonochem. 2: S115–S121.
45. Hui, Y. H. 2006. Handbook of Food Science, Technology, and Engineering. Vol. I. CRC Press. Boca Raton, FL.
46. Hunt, J. V., J. A. Simpson, and R. T. Dean. 1988. Hydroperoxide-mediated fragmentation of proteins. Biochem. J. 250: 87-93.
47. Huppertz, T., A. L. Kelly, and P.F. Fox. 2002. Effects of high pressure on constituents and properties of milk. Int. Dairy J. 12: 561–572.

48. IFST. 1993. Shelf Life of Foods – Guidelines for its Determination and Prediction. Institute of Food Science and Technology. London, UK.
49. Ipsen, R., J. Otten, E. Dominguez, K. B. Qvist. 2000. Gelation of whey proteins induced by proteolysis or high pressure treatment. Aust. J. Dairy Technol. 55: 49-52.
50. Jayasooriya S. D., B. R. Bhandari, P. Torley, and B. R. D'Arcy. 2004. Effect of high power ultrasound waves on properties of meat: a review. Int. J. Food Prop. 7: 301-319.
51. Johnston, D. E. 1995. High pressure effects on milk and meat. Loughborough: Nottingham University Press. 99–121.
52. Kaminogawa, S., and K. Yamauchi. 1972. Acid protease of bovine milk. Agr. Biol. Chem. 26: 2351-2356.
53. Karatapanis, A. E., A. V. Badeka, K. A. Riganakos, I. N. Savvaidis, and M. G. Kontominas. 2006. Changes in flavour volatiles of whole pasteurized milk as affected by packaging material and storage time. Int. Dairy J. 16: 750–761.
54. Karatapanis, A. E., A. V. Badeka, K. A. Riganakos, I. N. Savvaidis, and M. G. Kontominas. 2006. Changes in flavour volatiles of whole pasteurized milk as affected by packaging material and storage time. Int. Dairy J. 16: 750–761.
55. Kelly, A. L., F. O'Flaherty, and P. F. Fox. 2006. Indigenous proteolytic enzymes in milk: A brief overview of the present state of knowledge. Int. Dairy J. 16:563-572.
56. Kivelä, T. 1996. Easier cheese mould cleaning by ultrasonics. Scandinavian Dairy Information. 10: 34-35.
57. Knorr, D., M. Zenker, V. Heinz, and D. U. Lee. 2004. Applications and potential of ultrasonics in food processing. Trends Food Sci. Techn. 15: 261-266.
58. Korycka-Dahl. M., B. Ribadeau-Dumas, N. Chene, and J. Martal. 1983. Plasmin activity in milk. J. Dairy Sci. 66: 704.
59. Lopez, P., F. J. Sala, J. L. de la Fuente, S. Condon, J. Raso, and J. Burgos. 1994. Inactivation of peroxidase, lipoxygenase and polyphenol oxidase by manothermosonication. J. Agric. Food Chem. 42: 252-256.
60. Lopez, P., A. Vercet, A. C. Sanchez, and J. Burgos. 1998. Inactivation of tomato pectic enzymes by manothermosonication. Z. Lebensm. Unters. For. 207: 249-252.

61. Madadlou, A., M.E. Mousavi, Z. Emam-djomeh, M. Ehsani, and D. Sheehan. 2009. Sonodisruption of re-assembled casein micelles at different pH values. Ultrason. Sonochem. 16: 644–648.
62. Makino, K., M. M. Mossoba, and P. Riesz. 1983. Chemical effects of ultrasound on aqueous solutions. Formation of hydroxyl radicals and hydrogen atoms. J. Physical Chemistry. 87: 1369–1377.
63. Malik, A. C., and A. M. Swanson. 1974. Heat-stable proteases from psychrotrophic bacteria in milk. J. Dairy Sci. 57:591.
64. Manas, P., B. Munoz, D. Sanz, and S. Condon. 2006. Inactivation of lysozyme by ultrasonic waves under pressure at different temperatures. Enzyme Microb Tech. 39: 1177-1182.
65. Martinez, F., I. Desai, A. Davidson, S. Nakai, and A. Radcliffe. 1987. Ultrasonic homogenization of expressed human milk to prevent fat loss during tube feeding. J. Pediatr. Gastroenterol. Nutr. 6: 593–7.
66. Mason, T. J., L. Paniwnyk, and J. P. Lorimer. 1996. The uses of ultrasound in food technology. Ultrason. Sonochem. 3: 253-260.
67. McMahon, D. J. 1996. Age-gelation of UHT milk: Changes that occur during storage, their effect on shelf life and the mechanism by which age-gelation occurs. Heat treatments and alternative methods. Int Dairy F. 9602. 315-325.
68. Murray, B. S. 1997. Equilibrium and dynamic surface pressure-area measurements on protein films at air-water and oil-water interfaces. Colloid Surface. 125: 73-83.
69. McClements D. J. 1995. Advances in the application of ultrasound in food analysis and processing. Trends Food Sci. Techn. 6: 293-299.
70. Meilgaard, M., G. V. Civille, and B.T. Carr. 1999. Sensory Evaluation Techniques. 4th ed. CRC Press, New York.
71. Mulder H., and P. Walstra. 1974. The milk fat globule: Emulsion science as applied to milk products and comparable foods. Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, UK.

72. Mussa, D. M., and H. S. Ramaswamy. 1997. Ultra high pressure pasteurization of milk: Kinetics of microbial destruction and changes in physico-chemical characteristics. Lebensm-Wiss Technol. 30: 551–557.
73. Newstead, D. F., G. Paterson, S. G. Anema, S. J. Coker, and A. R. Wewala. 2006. Plasmin activity in direct-steam-injection UHT-processed reconstituted milk: Effects of preheat treatment. Int. Dairy J. 16: 573-579.
74. Nguyen, N. H. A., and S. G. Anema. 2010. Effect of ultrasonication on the properties of skim milk used in the formation of acid gels. Innov. Food Sci. Emerg. Technol. 11: 616-622.
75. Ohmes, R. L., R. T. Marshall, and H. Heymann. 1998. Sensory and physical properties of ice creams containing milk fat or fat replacers. J. Dairy Sci. 81:1222–1228.
76. O'Sullivan, M. M., A. L. Kelly, and P. F. Fox. 2002. Influence of transglutaminase treatment on some physico-chemical properties of milk. J. Dairy Res. 69: 433–442.
77. Ordóñez, J. A., M. A. Aguilera, M. L. García, and B. Sanz. 1987. Effect of combined ultrasonic and heat treatment (thermoultrasonication) on the survival of a strain of *Staphylococcus aureus*. J. Dairy Res. 54: 61-67.
78. Ordóñez, A. I., F. C. Ibanez, P. Torre, Y. Barcina, and F. L. Perez-Elortondo. 1998. Application of multivariate analysis to sensory characterization of ewes' milk cheese. J. Sens. Stud. 13: 45–55.
79. O'Sullivan, M. M., A. L. Kelly, and P. F. Fox. 2002. Influence of transglutaminase treatment on some physico-chemical properties of milk. J. of Dairy Res. 69: 433–442.
80. Özbek, B., and K. Ülgen. 2005. The stability of enzymes after sonication. Process Biochem. 35: 1037–43.
81. Patist, A., and D. Bates. 2008. Ultrasonic innovations in the food industry: from the laboratory to commercial production. Innovative Food Sci Emerging Technol. 9: 147–54.
82. Pereda, J., V. Ferragut, J. M. Quevedo, B. Guamis, and A. J. Trujillo. 2007. Effects of ultra-high pressure homogenization on microbial and physicochemical shelf life of milk. J. Dairy Sci. 90: 1081–1093.

83. Pereda, J., V. Ferragut, M. Buffa, B. Guamis, and A. J. Trujillo. 2008. Proteolysis of ultra high pressure homogenised treated milk during refrigerated storage. Food Chem. 111: 696-702.
84. Phillips, L. G., M. L. McGiff, D. M. Barbano, and H. T. Lawless. 1995. The influence of nonfat dry milk on the sensory properties, viscosity, and color of low fat milks. J. Dairy Sci. 78: 1258–1266.
85. Politis, I., and K. F. Ng Kwai Hang. 1989. Environmental factors affecting plasmin activity in milk. J. Dairy Sci. 72: 1713-1718.
86. Politis, I., D.M. Barbano, and R.C. Gorewit. 1992. Distribution of plasminogen and plasmin in fractions of bovine milk. J. Dairy Sci. 75: 1402-1410.
87. Politis, I., B. Zavizion, D. M. Barbano, and R. C. Gorewit. 1993. Enzymatic assay for the combined determination of plasmin plus plasminogen in milk: Revisited. J. Dairy Sci. 76: 1260-1267.
88. Povey, M. J., and T. J. Mason. 1998. Ultrasound in Food Processing. Blackie Academic and Professional, New York.
89. Quiñones, H. J., D. M. Barbano, and L. G. Phillips. 1998. Influence of protein standardization by ultrafiltration on the viscosity, color, and sensory properties of 2 and 3.3% milks. J. Dairy Sci. 81: 884–894.
90. Ralyea, R. D., M. Wiedmann, and K. J. Boor. 1998. Bacterial tracking in a dairy production system using phenotypic and ribotyping methods. J. Food Prot. 61: 1336-1340.
91. Raviyan, P., Z. Zhang, and H. Feng. 2005. Ultrasonication for tomato pectinmethylesterase inactivation: effect of cavitation intensity and temperature on inactivation. J. Food Eng. 70: 189-196.
92. Riener, J., F. Noci, D. A. Cronin, D. J. Morgan, and J. G. Lyng. 2009. Characterisation of volatile compounds generated in milk by high-intensity ultrasound. Int. Dairy J. 19: 269–272.
93. Riesz, P., and T. Kondo. 1992. Free radical formation induced by ultrasound and its biological implications. Free Radical Bio. Med. 13: 247–270.

94. Reinemann, D.J., G. A. Mein, D. R. Bray, D. Reid, and J. S. Britt. 1997. Troubleshooting high bacteria counts in farm milk. Troubleshooting high bacteria counts in farm milk. Proceedings 36th Annual Meeting, National Mastitis Council, Inc., Albuquerque, NM. 65-79.
95. Roberts, R. T. 1993. High intensity ultrasonics in food processing. Chem. Ind. 15: 119-121.
96. Roland, A. M., L. G. Phillips, and K. J. Boor. 1999. Effects of fat replacers on the sensory properties, color, melting, and hardness of ice cream. J. Dairy Sci. 82: 2094–2100.
97. Rollema, H. S., S. Visser, and J. K. Poll. 1983. Spectrophotometric assay of plasmin and plasminogen in bovine milk. Milchwissenschaft. 38: 214-217.
98. Rollema, H.S., and J. K. Poll. 1986. The alkaline milk proteinase system: kinetics and mechanism of heat inactivation. Milchwissenschaft. 41: 536-540.
99. Sakakibara, M., D. Wang, R. Takahashi, K. Takahashi, and S. Mori. 1996. Influence of ultrasound irradiation on hydrolysis of sucrose catalyzed by invertase. Enzyme Microb Technol. 18: 444–448.
100. Simal S., J. Benedito, G. Clemente, A. Femenia, and C. Rosselló. 2003. Ultrasonic determination of the composition of a meat – based product. J. Food Eng. 58: 253-257.
101. Simon, M., and A. P. Hansen. 2001. Effects of various dairy packaging materials on the shelf life and flavor of pasteurized milk. J. Dairy Sci. 84: 767–773.
102. Sorhaug, T., and L. Stepaniak. 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends Food Sci. Techn. 8: 35-41.
103. Tetra Pak. 2000. Handbook of Dairy Processing. Tetra Pak Processing Systems AB, Lund, Sweden.
104. Toma, S., A. Gaplovsky, and J. L. Luche. 2001. The effect of ultrasound on photochemical reactions. Ultrason. Sonochem. 8: 201–207.
105. USDA. 2009. Dairy background: From raw milk to dairy products. Available: http://www.ers.usda.gov/briefing/dairy/background.htm. Accessed on 07/05/2011.

106. USDA. 2011. Milk: Production by year, US. Available: http://www.nass.usda.gov/Charts_and_Maps/Milk_Production_and_Milk_Cows/milkprod.asp. Accessed on 07/05/2011.

107. van Boekel, M. A. J. S. 1998. Effect of heating on Maillard reactions in milk. Food Chemistry. 62: 403–414.

108. Vercet, A., P. Lopez-Buesa, and J. Burgos. 1999. Inactivation of heat-resistant pectin methylesterase from orange by manothermosonication. J. Agric. Food Chem. 47: 432-437.

109. Vercet, A., J. Burgos, and P. Lopez-Buesa. 2002. Manothermosonication of heat-resistant lipase and protease from *Pseudomonas fluorescens*: effect of pH and sonication parameters. J. Dairy Res. 69: 243-254.

110. Vega-Mercado, H., J. R. Powers, G. V. Barbosa-Canovas, B. G. Swanson. 1995. Plasmin inactivation with pulsed electric fields. J. Food Sci. 60: 1143-1146.

111. Villamiel, M., and P. de Jong. 2000. Influence of high-intensity ultrasound and heat treatment in continuous flow on fat, proteins, and native enzymes of milk. J. Agri. Food Chem. 48: 472-478.

112. Villamiel, M., and P. de Jong. 2000. Influence of high-intensity ultrasound and heat treatment in continuous flow on fat, proteins, and native enzymes of milk. J. Agri. Food Chem. 48: 472-478.

113. Walstra, P., and R. Jenness. 1984. Rheological properties in Dairy Chemistry and Physics. John Wiley and Sons, New York, NY. 290–300.

114. Wrigley, D. M., and N. G. Lorca. 1992. Decrease of *Salmonella typhimurium* in skim milk and egg by heat and ultrasonic wave treatment. J. Food Prot. 55: 678-680.

115. Wu, H., J. G. Hulbert, and J. R. Mount. 2001. Effects of ultrasound on milk homogenization and fermentation with yogurt starter. Innov. Food Sci. Emerg. Technol. 1: 211-218.

116. Zheng L., and D. W. Sun. 2006. Innovative applications of power ultrasound during food freezing processes – a review. Trends Food Sci. Techn. 17: 16-23.