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The effects of high power ultrasonic energy on milk plasmin activity

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The effects of high power ultrasonic energy on milk plasmin activity

by

Cindu Annandarajah

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Agricultural and Biosystems Engineering

Program of Study Committee:
David Grewell, Major Professor
Stephanie Clark
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Iowa State University
Ames, Iowa
2015

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DEDICATION

To the cows that give so much for science and yet expect nothing in return, including research findings, you are loved.
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NOMENCLATURE

PG  Plasminogen
PIs  Plasmin inhibitors
PAs  Plasminogen activators
PAIs  Plasminogen activator inhibitors
LTLT  Low temperature long time
HTST  High temperature short time
UP  Ultra pasteurized
UHT  Ultra High pasteurized
PS  Psychrosonication
p-p  Peak to peak
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ABSTRACT

The shelf life of pasteurized milk is limited by heat-stable proteases that undergo depolymerization, causing age gelation and bitterness. Proteolysis of milk during storage can be attributed to both native proteases and the proteases produced by bacteria, such as plasmin, which is the major native protease in milk. Ultra-high temperature (UHT) processing (>135°C for 2 s) inactivates proteases and extends shelf life up to 9 months but adversely affects sensory properties. Ultrasound is an emerging non-thermal food processing technology that is being explored as an alternative to pasteurization, as it minimize flavor loss, facilitates homogenization, and saves energy compared to thermal processing. A preliminary study by Vijayakumar et al. has shown that thermosonation (72°C, 152 µm peak-to-peak (p-p) for 3 min) inactivated proteases without producing off-flavor.

With the aim of inactivating plasmin, the present study evaluated the feasibility of extending milk shelf life by combining short durations (≤ 60 s) sonication as an adjunct to pasteurization. We refer to this approach as thermosonication to reflect its use of both high-power ultrasound and thermal treatment. Batch thermosonication was conducted using a bench scale ultrasonic unit operating at a frequency of 20 kHz and a maximum output of 2.2 kW. Skim milk was sonicated at short duration with constant amplitude of 170 µm (p-p), 72°C for 10, 30 and 60 s. The enzyme activity of raw, pasteurized, and thermosonicated milk was analyzed by total plasmin assay. A plasmin activity reduction of 83 and 96% up to day 49 was observed for both 30 and 60 s sonication times. Because another preliminary study by Benner et al. reported lower total aerobic bacterial count (TAC) via psychrosonication with subsequent pasteurization, the latter process was tested for scale-up potential in a continuous flow system.
An ultrasonic continuous flow process was tested using a high power flow-through “donut” horn. The donut horn, which vibrates radially, was placed inside a 5-L sonication reactor. The amplitude was maintained at 12 μm\textsubscript{p-p} and the feed flow rate was varied between 4 and 6 L/min. Samples that were treated with psychrosonication only showed no difference in plasmin activity compared to raw milk. However, milk that was psychrosonicated and pasteurized showed significant lower plasmin activity than raw milk. No significant difference was found in plasmin activity between control pasteurized and psychrosonicated samples followed by pasteurization, thus confirming that the major reduction of plasmin activity is a result of the pasteurization.

Based on these results, thermosonication is a promising pretreatment method for shelf life extension as an adjunct to pasteurization. However, our methods to translate the shelf life extension process through psychrosonication, under the conditions of this study, did not offer a feasible technology to be used in the dairy industry to extend milk shelf life.
CHAPTER 1
INTRODUCTION AND OBJECTIVES

1.1. Introduction

For thousands of years, milk has been an important part of the human diet. It is also considered unique because there are no other beverages in the world that contain as many natural nutrients and health benefits as milk. The natural vitamins and minerals present in milk include calcium, niacin, phosphorus, potassium, protein, riboflavin, vitamins A, B12, and D. Thus, milk is used extensively by nearly every region of the world, and especially to feed infants. In its various forms, such as whole milk, reduced fat, skim, and non-fat milk as well as lactose free, milk plays an important role in growth, energy source, maintenance and repair and appetite satisfaction of humans [1]. With a growing population and increase in demand in the United States, milk production has seen an increase of nearly 50% despite the decrease in the number of cows from 12 million to 9 million over the last 37 years [2]. Despite the fact that milk is produced widely in the US, only one-third of the milk is processed into fluid milk for consumption, while the balance goes to production of other dairy by-products such as cheese, ice cream, and butter [2].

The United States produces the second highest quantity of raw cow milk, followed by China and Brazil [3, 4]. However, per capita consumption of milk in this country has gradually decreased by 36% over the last 40 years [5]. The decline is attributed to various reasons: the tough competition of highly perishable fluid milk in the marketplace against shelf stable beverages (i.e., juices, soda, etc.) [5], wrong perception of milk’s nutritional
value, especially the fat and cholesterol content, convenience, and cost of fluid milk [6]. One of the ways to effectively compete and sustain the position in the beverage market is by extending the shelf life of the fluid milk that allows extended geographical coverage of product distribution.

Typically, raw milk is pasteurized through conventional thermal pasteurization techniques to lower the spoilage rate and to eliminate the pathogens to safe levels for normal storage (refrigeration) of approximately 21 days. However, heat resistant spoilage bacteria and proteases limit milk shelf life because they are able to survive thermal treatment; proteases can cause milk deterioration that affects its quality and flavor. Although ultra-high temperature (ultra-pasteurized) processing completely eliminates the bacteria and inactivates some proteases, milk quality is detrimentally affected in terms of flavor and appearance (sensory properties). With the aim of extending shelf life of milk while maintaining its sensory properties, this study proposes short time thermosonication, a combination of high temperature short time (HTST) pasteurization and ultrasonication.

Ultrasound is defined as sound waves with frequencies beyond the human hearing threshold (above 20 kHz) [7]. When power ultrasound is applied in a fluid medium, the sound waves propagate in alternate compression and rarefaction pressure regions and create cavitation. Cavitation is the formation of vapor-filled cavities or microbubbles in liquid when it is subjected to negative pressures. These microbubbles grow unstable as they increase in size and collapse drastically, releasing shock waves (mechanical or shear forces) resulting in physical and chemical changes. During ultrasound propagation in liquid, continuous displacement of liquid movement generates acoustic streaming that helps in mixing and enhances energy distribution in the medium. This technology has been used in a wide range
of chemical, biological, medical, and industrial fields for various applications. Preliminary study shows that thermosonication (72°C, 152 μm for 3 min) inactivates microorganisms and proteases without producing off-flavors [8]. Additionally, research by Manas et al. [9], Raviyan et al. [10], and Villamel and DeJong [11] demonstrated inactivation of enzymes in food using heat and high-power ultrasound. In these studies, thermosonication exhibited the potential to be an adjunct to pasteurization to extend milk shelf life by inactivating the protease enzymes.

1.2. Objectives

The overall objective of this research is to test the feasibility of integrating ultrasound as an adjunct to pasteurization to extent milk shelf life. This research includes the investigation of the total plasmin activity on skim and whole milk treated by thermosonication. Plasmin is an indigenous proteinases found in milk that is responsible for proteolysis in milk that results in age gelation and off-flavors.

Following are detailed objectives of the thesis and brief descriptions of the respective approaches to the research:

1. Determine if thermosonication reduces total plasmin activity in skim milk effectively throughout six weeks of shelf-life.

2. Determine if psychrosonication, followed by pasteurization, reduces total plasmin activity in whole milk effectively throughout six weeks of shelf-life.
1.3. Thesis Organization

This thesis is divided into five chapters:

1. Chapter 1 includes introduction and objectives.
2. Chapter 2 includes literature review.
3. Chapter 3 is a journal paper, titled “Impact of batch thermosonication on plasmin activity in stored skim milk: time-amplitude effects”.
4. Chapter 4 is also a journal paper, titled “Impact of continuous sonication followed by pasteurization at industrially relevant flow rates on plasmin activity in stored whole”.
5. Chapter 5 includes general conclusions from Chapter 3 and Chapter 4.
2.1. Standard of Identity of Milk

Animal milk is a nutritious dairy product that has been consumed by humans since historical times, as it is used to feed the mammalian offspring. According to the United States Code of Federal Regulations, “milk is the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows” [12]. The code also states that the milk that is in its final package for drinking, intended for shipment across state lines, must be pasteurized or ultrapasteurized and needs to have a minimum of 8.25% milk solids not fat, and at least 3.25% milkfat. In addition, the milkfat content can be added or separated to modify the milk for cream, concentrated milk, dry whole milk, skim milk, concentrated skim milk, or nonfat dry milk. In this standard of identity, milk may be homogenized, and other optional ingredients, such as vitamins A and D, sweetener, emulsifiers, stabilizers, fruit and fruit juices, and natural and artificial flavorings may be added.

2.2. Milk Constituents

Generally, cow’s milk in the United States is 87.7% water. The balance of the total solids is comprised of 3.4% fat and protein, 4.8% carbohydrate and 0.7% minerals that are also referred to as ash [4]. Various factors, such as the specific cow, its breed, feed, lactation stage, and age could result in the variation in milk constituents. Irrespective of the
environmental conditions and variations in herd management, the average milk composition remains relatively consistent in the United States [3].

2.3. Pasteurization

2.3.1. Food Laws

The Grade “A” Pasteurized Milk Ordinance (PMO), regulated by the Food and Drug Administration (FDA), is a set of standards for the production, processing, and packaging of Grade “A” milk. This ordinance requires milk products to be pasteurized according to minimum processing conditions before the milk is sold for human consumption [13]. This regulation is a safety measure that is required to eliminate pathogenic microorganisms such as Listeria monocytogenes, Coxiella burnettii, Yersinia enterocolitica, Campylobacter jejuni, and Escherichia coli, which represent significant health hazard to consumers [14]. Thus, the legal time and temperature for milk pasteurization is standardized mainly to reduce pathogenic bacteria to a safe level and also to maintain milk quality (by killing many spoilage microorganisms), thus extending shelf life of milk [4].

2.3.2. Low Temperature Pasteurization

Low temperature long time (LTLT) pasteurization describes the process of heating the milk to a relatively low minimum temperature (62.8°C), and holding it there for at least the minimum time (30 min) required by law before it is rapidly cooled. This pasteurization is typically completed in a vat, in a batch process. In contrast, in the continuous process also known as high temperature short time (HTST) pasteurization, milk is heated to 145°F
(71.7°C) and held for no less than 15 seconds, followed by rapid cooling. Both of these conditions are the mildest form of pasteurization used in the dairy industry and have proven to inactivate the most heat resistant organisms, namely *Mycobacterium bovis* and *Coxiella burnetti*, found in milk. These pasteurization processes kill all pathogens, most of the spoilage bacteria, and many indigenous enzymes, thus extending milk shelf life [15]. However, some thermoduric microorganisms, such as *Microbacterium spp.*, *thermophilic streptococci*, and bacterial spores can survive pasteurization. In addition, native and bacterial spoilage enzymes can be inactive for a short time after pasteurization and regenerate upon storage. This rather mild treatment does not affect milk quality, and the serum protein and bacteriostatic properties remain unchanged [15]. Milk composition dictates the pasteurization conditions required. For example, the pasteurization temperature needs to be increased by 5°F (3°C) if the milk contains more than 10% fat, contains added sweeteners, or if it is concentrated [16].

### 2.3.3. High Temperature Pasteurization

When raw milk is subjected to severe pasteurization, the microorganisms and spoilage enzymes are completely eliminated through sterilization. This process is the basis of ultra-pasteurization (UP), which is completed by heating the milk to 280°F (137.7°C) for 2 s. Milk processed by UP has longer shelf life, but requires refrigeration. Ultra high temperature (UHT) pasteurization involves heating milk to the same temperature as UP, but packaging, aseptically, in sterile packages. UHT milk is shelf stable and does not require refrigeration until opened. Even though this method does not allow recontamination during processing,
UHT pasteurization has a detrimental effect on milk quality and also results in off-flavors as a result of Maillard reaction and the release of sulfur compounds in milk [4].

2.3.4. Pasteurization Effects on Milk

Pasteurization increases milk safety by destroying pathogens and extends its shelf life. However, pasteurization at higher levels than conventional pasteurization processes may result in changes in functionality, flavor, color, and viscosity of milk. For instance, during UHT pasteurization, the lactose (carbohydrate) reacts with lysine (protein) in the milk and this interaction causes Maillard reaction or browning of the milk. In addition to producing off-flavors and change in color, this reaction also detrimentally affects the nutritive value, as the lactose-lysine reaction becomes irreversible [17]. Also, high temperature pasteurization can also cause cross linkage between β-lactoglobulin, the major whey protein responsible for binding water, and κ-casein, which accelerates age gelation [18].

2.4. Milk Shelf Life

The food industry has a major responsibility in maintaining food safety and sensory quality to meet consumers’ increasing expectations regarding the quality of fluid milk with longer shelf life from the time of purchase to consumption. The definition of shelf life has changed over the years to meet a more practical characterization. The Institute of Food Science & Technology (IFST) Guidelines (1993) defined shelf life as the “time during which the food product will remain safe while retaining its desired sensory, physical, chemical, and biological properties and comply with any label declaration of nutritional data.” Raw milk can remain fresh for approximately 5 to 7 days when kept refrigerated at 4°C. In the case of
raw milk, the shelf life is typically determined by the collection and handling process, cleanliness of milking environment, and its microbial and somatic cell count [4, 19].

Milk shelf life is limited mainly by psychrotrophs, a type of bacteria produced as a result of post-processing contamination. These bacteria contain both gram-negative and gram-positive rod-shaped species that account for more than 90% of the total microbial population in cold raw milk [19, 20]. Both HTST and LTLT pasteurization can typically stabilize milk for approximately 14 to 21 days when packaged and stored under refrigerated conditions [5, 21]. In these processed milks, the end of shelf life occurs when the psychrotropic bacteria level reaches $10^6$ CFU/ml, producing off-flavors as a result of increasing microbial activity and metabolism [5, 20, 22]. These psychrotropic bacteria are able to multiply at low temperatures and produce heat stable proteases and lipases that cause spoilage by coagulating milk protein and increasing the amount of free fatty and amino acids [20]. In addition, the thermotrophic endospores of gram-positive bacteria such as Bacillus spp., Peanibacillus, and Microbacterium survive the pasteurization processing and serve as the predominant cause of microbial spoilage in HTST pasteurized milk after 17 days of refrigerated storage [5]. The ability of psychrotrophs to produce living organisms and heat stable bacteria in any conditions is the main reason for the reduction in milk shelf life and quality [20]. Thus, these heat-resistant psychrotropic contaminants need to be eliminated for further shelf life extension of fluid milk. Ultra-pasteurization and UHT pasteurization completely kill the bacteria and extend the shelf life up to 6 months. However, consumers, especially children, dislike the off-flavor in the milk caused by extreme heating and prefer HTST pasteurized milk [21].
2.4.1. Shelf Life Limiting Proteases

Proteases are one of the major enzymes in milk that causes a reduction in milk shelf life by promoting depolymerization of proteins into peptides through proteolysis. Proteolysis causes age gelation, which affects the quality of milk. This process can be attributed to both native proteases and also by psychrotroph-produced proteases that survive pasteurization and grow during refrigerated storage. At a temperature of 20°C, psychrotrophs, especially *Pseudomonas*, produce twice the amount of proteases and lipase than at a temperature of 5°C. In addition to being able to reproduce at a wide range of temperatures, (5°C - 45°C), these proteases are highly heat stable and can withstand UHT pasteurization (140°C for 4s). After surviving extreme pasteurization, proteases from psychrotrophs hydrolyze the casein proteins mainly α-, β-, and κ-casein. The depolymerization of κ-casein results in bitterness of the milk [18, 23-25].

2.4.2. The Plasmin System

Plasmin (EC 3.4.21.7) is the principal indigenous proteinase found in milk apart from cathepsins and elastase. The plasmin system (Figure 2.1) is composed of plasmin, its zymogen (plasminogen), plasmin inhibitors, plasminogen activators, and plasminogen activator inhibitors.

![Figure 2.1 The plasmin system and its function](image-url)
Most of the plasmin is present as inactive plasminogen and is largely associated in the casein micelle of the milk. Plasminogen activators (PAs) together with urokinase convert inactive plasminogen to active plasmin by cleaving the Arg\textsuperscript{557}-Ile\textsuperscript{558} peptide bond, and this conversion results in protein depolymerization in milk upon storage. The ratio of plasminogen to plasmin in milk has been reported to vary from 50:1 to 2:1. During protein depolymerization, plasmin cleaves the carboxyl side of \textalpha-\text{Lys} and \textalpha-\text{Arg} residues [25]. Most of the plasmin depolymerization takes place actively in the casein and very little to almost none in the whey protein [25]. The two components that are able to cease the plasmin system, plasminogen activator inhibitors and plasmin inhibitors, are relatively heat sensitive. However, plasminogen, plasminogen activator, and plasmin are extremely heat stable. Thus, increased plasmin activity can be observed in stored pasteurized milk as a result of the inactivation of plasmin and plasminogen inhibitors [23, 25]. Highest activity of bovine milk plasmin is observed at pH 7.5 to 8.0 and at 37°C [23]. Having similar stability to heat, plasmin and plasminogen not only survive the pasteurization process (72°C for 15 s) completely at pH 6.8, but have also been shown to be mildly active after UHT processing treatments [26]. Chen et al. (2003) reported that complete inactivation of plasmin enzyme in milk occurs only when the milk is heated to 120°C for 15 min [27]. However, processing milk at such extreme conditions results in off-flavors in milk and affects consumers’ acceptance of the product. Thus, new technologies, such as high-pressure processing, pulsed electric field, irradiation, and ultrasound are being explored as potential methods to inactivate the shelf life limiting enzymes, in particular plasmin.
2.4.3. Characterization of Plasmin Activity Using Total Plasmin Assay

Total plasmin activity is defined as the activity when inactive plasminogen is converted to active plasmin in milk or cream samples [30]. The procedure for measuring the total plasmin activity is based on the methodology used by Politis et al. [28], which was a modification of their conventional methodology based on Rollema et al. [29]. An aliquot of milk samples of 5 ml is centrifuged at 2,000 × g for 15 min to separate cream and skim milk. The cream layer is discarded and skim milk is ultracentrifuged at 100,000 × g for 1 h at 4°C to separate the supernatant (milk serum) and the pellet (casein) fractions. This process ensures the plasmin and plasminogen remain intact in the casein micelles. The milk serum is removed from the casein and 50 mM of Tris buffer (pH 8.0) containing 110 mM NaCl and 50 mM ε-aminocaproic acid (EACA) are added to reconstitute to the original volume. The casein pallet is then resuspended in the buffer solution and incubated at room temperature (20°C ± 3°C) for 2 h to ensure the dissociation of plasmin and plasminogen from casein and the transfer to the buffer. Following the incubation, the casein micelles solution is centrifuged again at 100,000 × g for 1 h at 4°C to allow the transfer of plasmin and plasminogen into the buffer.

For the absorbance analysis, 50 µl of supernatant buffer containing transferred plasmin and plasminogen are mixed to 950 µl of 50 mM of Tris buffer (pH 7.4) containing 110 mM NaCl, 0.6 mM H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride and 2.5 mM EACA. Another 150 plough units of urokinase are added to this solution to convert inactive plasminogen to active plasmin. The reaction mixture is incubated for 60 min at 37°C for the sufficient conversion of plasminogen to plasmin [30]. The absorbance of conversion at 405 nm is measured using a spectrophotometer at every 30 min interval for 3 h. A similar
buffer reaction mixture without the milk serum fraction serves as the blank. Absorbance is averaged for three replications, plotted against time, and the rate of absorbance increase (p-nitroanilide formation) is calculated. The rates of p-nitroanilide formation were determined by calculating the regression of plasmin activity over time. From the slopes of the regression lines, the percentage of plasmin activity ($%_{plas.activity}$) after treatment (heat and thermosonication) was calculated in Equation (2.1) as follows:

$$%_{plas.activity} = 100 \frac{s_{treated\ sample}}{s_{control}}$$  \hspace{1cm} (2.1)

where $s_{treated\ sample}$ is the slope of the treated sample and $s_{control}$ is the slope of control.

### 2.5. Ultrasound

Ultrasound is defined as sound waves or vibrations with frequencies beyond the human hearing limit, usually above 20 kHz [7]. Ultrasonic units operate with frequencies varying from 20 kHz to a few gigahertz depending of the application range. When power ultrasound is applied in a fluid medium, the sound waves propagate in alternate compression and rarefaction pressure regions and create cavitation (Fig. 2.2).

![Wave propagation and particle displacement](image)

**Fig 2.2** Compression and rarefaction of ultrasound waves (Source: Silvana, Martini [32])
To create a fundamental effect on the liquid medium, ultrasound uses acoustic pressure ($P_a$) in addition to the existing hydrostatic pressure. The acoustic pressure is created by sound waves and can be described by Equation 2.2: [31]

$$P_a = A \sin(2\pi ft) \quad (2.2)$$

The acoustic pressure ($P_a$) is a sinusoidal wave and is dependent on:

- Amplitude, $A$. Amplitude is the peak oscillation displacement of the sound wave. It is the maximum pressure amplitude of the wave and is directly proportional to the power input of the transducer.
- Frequency, $f$. The angular frequency of a sound wave is the number of waves that pass through a point at a certain time (s).
- Time, $t$. The amount of time (s) taken to apply the sound waves through a medium.

Ultrasound can be divided into two frequency ranges: low and high power ultrasound. The possible frequency of ultrasonic waves is generally inversely proportional to the power. High power ultrasound is typically generated at low frequencies, ranging from 16 to 100 kHz. At lower frequencies, cavitation bubbles can cause physical and chemical changes in the liquid medium. Thus, power ultrasound can be used for cell disruption, emulsification, cleaning, drilling, and welding. High frequency ultrasound has been applied in the food science research area, from monitoring carbohydrate and lipid crystallization, characterizing edible oils and fats, predicting viscoelastic properties of materials, to characterizing emulsions and suspensions [32].

In contrast, diagnostic ultrasound is commonly used for imaging in the medical field, as it measures velocity and reflects the absorption coefficient of waves passing through a
medium. This type of ultrasound uses high frequencies, extending from 1 to 10 MHz, low power (< 10 mW/cm²) and is also non-destructive. Diagnostic ultrasound cannot be used to produce cavitation effects, as at such high frequencies, the pressure is too low, thus obstructing the formation of microbubbles. This characteristic makes them suitable for diagnostic application such as medical scanning, stain treatments and dentistry, as they do not cause any physiochemical changes. However, it should be noted that high power ultrasound, higher intensity of diagnostic ultrasound, is also used in the medical field to modify body tissues such as cancer and stone treatment [32, 33].

2.5.1. Cavitation

Cavitation is the formation of vapor-filled cavities or microbubbles in liquids when they are subjected to negative pressure. When passed through a liquid medium, ultrasound creates a cyclic succession of compression and expansion (rarefaction). Gradual oscillation creates positive pressure during the compression cycle that compresses the gasses. In contrast, negative pressure during the expansion cycle causes the small vapor-filled voids to grow in the liquid, which are known as cavitation bubbles. There are two types of cavitation: transient (inertial) cavitation and stable (non-inertial) cavitation [32]. During this non-linear acoustical vibration, a stable cavitation bubble oscillates around its own radius of equilibrium, resulting in smaller bubbles around to cause strong eddies in the liquid. However, a transient bubble increases its radius progressively beyond its equilibrium radius before collapsing drastically at surface velocity of 103 m/s. Consequently, an extreme amount of energy density with localized high temperature and pressure is released that assist chemical reactions. The microbubbles from stable cavitation can lead to transient cavitation
whereas the fragments of microbubbles released during transient cavitation could undergo stable cavitation, thus making the cavitation characterization vague. Cavitation consists of the repetition of three distinctive phases: formation (nucleation), rapid growth (expansion) of the bubble to a critical size, and a violent collapse in the medium [33].

2.5.2. Acoustic Streaming

Acoustic streaming became well-known in 1978, after Sir James Lighthill provided a detailed model based on first order principles that corrected previous misconceptions [35]. He explained that acoustic streaming is a consequence of gradients of momentum and fluid current that are caused by the dissipation of acoustic energy. When ultrasound waves are applied to a liquid, the particles in the medium oscillate around equilibrium and generate a continuous displacement of liquid movement. This non-linear effect of converting high intensity sound energy to kinetic energy is known as acoustic streaming and is independent of the cavitation effect. Acoustic streaming is a combined influence of Eckart streaming, Rayleigh streaming, and Schlichting streaming, shown as Region 1, 2 and 3 respectively in Figure 2.3. Raleigh streaming by the solid-liquid boundaries permits good heat transfer at high speed that results in turbulence, thus helping in heat and mass transfer [36].
2.5.3. Ultrasonic Tooling Design

An ultrasonic system is essential for producing and transferring ultrasonic waves to the medium it is being applied to. Figure 2.4A shows the ultrasonic system used for a batch process, while Figure 2.4B is the set-up used for a continuous system. These systems consist of four basic components: power supply, converter, booster, and horn.

Figure 2.3 Illustration of acoustic streaming observed in liquid media. (Source: Chand et al. [37])

Figure 2.4 Ultrasonic equipment diagram (A) for batch process (Source: http://www.bransonultrasonics.com/) (B) for continuous process
The ultrasonic power supply converts AC line power to high frequency electrical energy. The power supply comes with features that allow users to control the sonication parameters. Ultrasound is then generated in the converter, which transforms the electrical energy to high frequency mechanical movement.

2.5.3.1. Converter

The electromechanical converter (transducer (motor)) contains piezoceramic discs produced from lead zirconate titanate (PZT) that are stacked together in Langevin sandwich form [37]. When electric charge is applied across the polarized ceramic discs, they expand/contract depending on polarity. The discs are stacked with electrodes between the discs and clamped using two metal surfaces to form a resonant system in which a metal central bolt clamps the system together. In this configuration the transducer converts electrical energy to an oscillating mechanical motion. The vibrational amplitude is restricted by the transducer’s maximum allowable stress, higher frequency, and other tooling design specifications. As the frequency increases, the power density (power per unit volume) of the converter increases. As a result, high frequency ultrasounds correspond to small converters and they heat more rapidly despite being 95-96% efficient in energy conversion [37]. Thus, higher frequencies tend to have lower maximum power capabilities.

2.5.3.2. Booster

The booster is threaded to the converter and increases, decreases, or transfers the amplitude by the transducer depending on the design and specification of the booster. These motions are then transferred to the horn. The booster is usually made of titanium or
aluminum alloy. The nodal plan of the booster can also serve as a location of securing/gripping the ultrasonic stack

2.5.3.3. Horn

The horn is usually made from materials that provide a good combination of acoustical and mechanical properties, usually titanium or aluminum alloys. When a horn vibrates at its resonant frequency, its ends move in opposite directions, expanding and contracting. The amplitude of the horn is measured as peak-to-peak displacement at the horn “face”. The function of the horn is to transfer the ultrasound energy to the liquid medium and its amplitude is adjusted by changing the mass ratio of the horn or by changing the input amplitude (at the converter). The ratio of a horn’s output amplitude to input amplitude is known as “gain” (ratio).

2.5.4. Ultrasound Application in Dairy Industry and Enzyme Inactivation

The use of ultrasound in the dairy industry is well known, although its industry-wide acceptance is relatively new compared to other processing methods such as using mechanical or thermal energy. Ultrasonic food processing is known to have a major impact on reducing the rate of processes, consuming only a fraction of time and energy normally required for conventional processes, with high reproducibility. In the dairy industry, ultrasound is used for cleaning whey-fouled membranes that are used for ultrafiltration and membrane separation [38]. Ultrasonic cutting of cheeses is gaining wide industry acceptance, replacing methods such as water jet cutting or using saws and knives, mainly because it increases hygiene [38]. The vibration produced during sonication prevents adherence of microorganisms and residual food on the tool which is repetitively cutting with high
precision, reducing product losses. In a study for ice cream production, ultrasonic treatment was used to enhance the nucleation rate and rate of crystal formation, which resulted in the formation of small crystals upon freezing [38]. Emulsification is an important process, especially for the homogenization of fluid milk and yoghurt. The cavitation during sonication effectively disrupts fat globules, resulting in small particle sizes and a more stable emulsion in shorter time than conventional homogenizing systems [38].

High power ultrasound is explored for inactivation of microorganisms in milk and other dairy products. Studies have shown increasing elimination of bacteria with increasing exposure time and intensity [39]. In addition, ultrasound, when combined with heat (thermosonication) or with heat and pressure (manothermosonication), leads to higher inactivation of microorganisms and enzymes [9, 11]. The studies also show that with high power ultrasound, a higher inactivation of enzymes is achieved in a shorter time compared to thermal inactivation alone [9, 11]. In contrast, Mason et al. [40], and Shah et al. [41] showed that at low intensity, ultrasound is capable of enhancing enzyme activity instead of inactivating them.

A novel application of ultrasound was implemented by scientists from Swinburne University of Technology in Australia and the Commonwealth Scientific and Industrial Research Organization (CSIRO) where they demonstrated large-scale milk fat separation using an ultrasonic separation technique. This technique has the potential to be integrated in the existing technology in the dairy industry. The cream separation is completed by using dual-plate transducers that are able to separate both large fat globules and small fat globules, resulting in better separation in shorter time than conventional methods [35].
Preliminary work by Vijayakumar et al. on the feasibility of integrating ultrasound into the pasteurization processing reported that thermosonication (152 µm_{p-p}, 3 min, 72°C) reduced plasmin activity by 94% in raw skim milk and by 96% in raw cream. In addition to the effective reduction in plasmin activity, thermosonication at 133 and 152 µm_{p-p}, 1 and 3 min also completely destroyed coliforms and over 99% of the total aerobic bacteria (TAC). More importantly, thermosonication did not induce off-aromas or viscosity changes, but inactivated microorganisms and protease enzymes.

These results showed promises of integrating ultrasound in the dairy industry for shelf life extension. However, it is not feasible to integrate the HTST unit in existing industrial processes with the processing times tested [8]. Thus, the current study will investigate the effect of short duration ($\leq$ 60 s) time-amplitude thermosonication on the plasmin activity of stored milk.
2.6. References

CHAPTER 3

IMPACT OF BATCH THERMOSONICATION ON PLASMIN ACTIVITY IN STORED SKIM MILK: TIME-AMPLITUDE EFFECTS

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3.1. Abstract

The effect of thermosonication on plasmin activity in skim milk stored up to 49 days was studied after treatment in a batch ultrasonic system operating at 20 kHz frequency. Fresh raw milk was centrifuged to obtain skim milk. In 100 mL batches, the skim milk was heated to 72°C for 15 s, followed immediately by sonication at 72°C, at a constant amplitude of 170 µm peak-to-peak (p-p) (approximately 140 W) for various times (10, 30, and 60 s). After thermosonication, the milk was stored at 4°C and the total plasmin activity of treated milk was analyzed on days 7, 21, 35, and 49 and compared with the controls (raw and pasteurized). Thermosonation inactivation of plasmin- and plasminogen-derived activity at 170 µm for 30 and 60 s decreased the total plasmin activity by 83 and 96%, respectively compared to raw milk up to day 49. Short duration (≤ 60 s) thermosonation was more effective than pasteurization in reducing plasmin activity.
3.2. Introduction

The United States is the second largest raw cow milk producing country in the world and accounts for 12% of the world’s production [1]. However, per capita consumption of milk in the US has decreased by 36% over the past 40 years [1, 2]. The decline can be associated with the tough competition of highly perishable fluid milk in the marketplace against shelf stable beverages such as soda, juice, and bottled tea that have captured a large portion of the beverage market in recent years. One of the ways to effectively compete and sustain milk’s position in the beverage market is by increasing the quality and extending the shelf life of fluid milk [3, 4].

One of the factors that detrimentally affect milk quality is proteolysis. Proteases are one of the major enzymes in milk that depolymerize proteins into peptides, and this process affects the quality of milk. Even though this enzymatic reaction is favorable in cheese ripening, as it helps in developing desired flavor, proteolysis causes age gelation and off-flavors in pasteurized fluid milk upon storage [5, 6]. Proteolysis can be attributed to native proteases and those from psychrotrophs that survive pasteurization. Plasmin (EC 3.4.21.7) is one of the principal indigenous proteinases found in milk that is responsible for proteolysis. The plasmin system is composed of plasmin, its zymogen, plasminogen (PG), plasmin inhibitors (PIs), plasminogen activators (PAs), and plasminogen activator inhibitors (PAIs). The two components that are able to shut down the plasmin system, PAIs and PIs, are quite heat sensitive, whereas PG, PAs, and plasmin are extremely heat stable. Thus, increased plasmin activity can be observed in stored pasteurized milk as a result of the inactivation of PAIs and PIs [7-10]. Ultra pasteurization (UP) and ultra high treatment (UHT) pasteurization kill nearly all of the bacteria, however, 30 to 40% plasmin activity is observed after these
processing conditions [9]. In order to inactivate plasmin completely, milk has to be heated to 120°C for 15 min [11- 13]. However, processing milk at such extreme conditions can result in off-flavors and can affect the consumers’ acceptance. Thus, new technologies, such as high-pressure processing, pulsed electric field, irradiation, and ultrasound are being explored as potential methods to inactivate the shelf life limiting enzymes, in particular plasmin.

Ultrasound is acoustic vibration at frequencies beyond the human hearing threshold, ranging from 18 - 20 kHz [14]. When high power ultrasound is applied in a fluid medium, cavitation occurs. Cavitation is the formation of vapor-filled cavities or microbubbles in liquid when it is subjected to negative pressures. These microbubbles increase their radius progressively beyond their equilibrium radius before collapsing drastically, resulting in high energy density dissipation with localized high temperature and pressure. Coakley et al. [15] investigated the effect of sonication alone on the inactivation of alcohol dehydrogenase, catalase, and lysozyme and reported exponential inactivation of more than 50% for alcohol dehydrogenase and lysozyme. In contrast, other researchers have observed higher inactivation of microorganisms and enzymes when ultrasound was combined with factors such as heat (thermosonication), or with heat and pressure (manothermosonication) [16, 17]. These studies also show that high power ultrasound achieves higher inactivation of enzymes in a shorter time than thermal inactivation alone. A study using thermosonication, a combination of high temperature short time (HTST) pasteurization and ultrasonication at 152 µm_{p-p} for 3 min at 72°C showed a decrease of 94% plasmin activity in raw skim milk and cream without affecting the sensory quality of the milk [18]. However, the long sonication time makes this method unfeasible in the dairy industry, as the HTST process typically takes
only 15 s. The purpose of this study was to examine the effect of short duration (≤ 60 s) thermostonication on plasmin activity.

3.3. Materials and Methods

3.1 Milk Preparation

Fresh raw whole milk was collected from the bulk tank of Iowa State University’s Dairy Farm (Ames, IA). Raw whole milk was transported to the Iowa State University Center for Crops Utilization Research (CCUR) pilot plant and the milk was separated into skim milk and cream within an hour of procurement. The milk separation process was completed using a centrifugal cream separator (Varidrive Motor, US Electrical Motors, Inc, Milford, CT; 1750 rpm); the skim milk was collected in a sterile container while the cream was discarded. Skim milk was refrigerated at 4°C for approximately an hour before treatment.

All of the chemicals used were of analytical grade. Tris buffer (pH 8.0) and NaCl were purchased from Fisher Scientific (Fair Lawn, NJ). Trizma buffer (pH 7.4), H-d-valyl-l-leucyl-l-lysine-p-nitroanilide dihydrochloride (VALY, V7127), ε-amino caproic acid (EACA), and urokinase were purchased from Sigma Aldrich (St. Louis, MO). Thinwall, Ultra-Clear™ ultracentrifuge tubes (5ml, 13 × 51mm) were purchased from Beckman Coulter (Brea, CA).

3.3.2. Batch Thermosonication Experiments

Two sets of control samples were prepared. Raw skim milk was stored in centrifuge tubes as control raw samples. For control pasteurized samples, 100 ml of raw skim milk was heated in a covered stainless steel bowl using a hot plate set to 300°C. The milk was stirred at
an interval of one minute using a sanitized glass rod to prevent foaming and scum formation. The milk was heated to 72°C and held for 15 s, mimicking pasteurization before storing in centrifuge tubes.

All skim milk, except the controls, were pre-heated in the same way as the control pasteurized samples and then sonicated using a Branson 2000 Series bench-scale ultrasonic unit (Branson Ultrasonics, Danbury, CT). The system is capable of operating at a maximum power output of 2.2 kW at a frequency of 20 kHz, as illustrated in Figure 3.1. The ultrasonic treatments were completed in a series of the Branson model 250 rosette cooling cells for Branson Sonifiers (model 201-123-003) immersed in water bath at 72 ± 2°C. Milk was sonicated at a constant amplitude of 170 µm ($p-p$) (average power: 140 W) for 10, 30, and 60 s and the corresponding energy densities and temperatures of three sonication times are reported in Table 3.1. The ultrasonic booster (Branson Ultrasonics, Danbury, CT) was a booster with 1.5 gain and the horn (Branson Ultrasonics, Danbury, CT) was a 20 kHz half-wavelength catenoidal titanium with a flat 13 mm diameter face (gain = 8). The horn was submerged 3 cm into the milk during sonication. The whole experiment was repeated three times over 12 weeks using new milk samples for each experiment.

![Figure 3.1 Batch process ultrasonic equipment diagram (Source: http://www.bransonultrasonics.com/)](http://www.bransonultrasonics.com/)
Table 3.1. Thermosonication treatment settings, average temperature before and after treatment, and average energy density.

<table>
<thead>
<tr>
<th>Treatment Type</th>
<th>Amplitude (µm)</th>
<th>Power (W)</th>
<th>Treatment Times (s)</th>
<th>T&lt;sub&gt;i&lt;/sub&gt; (°C)</th>
<th>T&lt;sub&gt;Paste&lt;/sub&gt; (°C)</th>
<th>Mean Energy Density of past. (J/ml)&lt;sup&gt;*&lt;/sup&gt;</th>
<th>T&lt;sub&gt;TS&lt;/sub&gt; (°C)</th>
<th>Mean Energy Density of US (J/ml)</th>
<th>Total Energy Density (J/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Raw</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>5.3</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Control Past</td>
<td>n/a</td>
<td>n/a</td>
<td>15</td>
<td>6.9</td>
<td>73.3</td>
<td>27.6</td>
<td>n/a</td>
<td>n/a</td>
<td>27.6</td>
</tr>
<tr>
<td>Thermo-sonicated</td>
<td>170</td>
<td>140</td>
<td>10</td>
<td>5.7</td>
<td>71.2</td>
<td>27.2</td>
<td>71.5</td>
<td>14.5</td>
<td>41.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>5.9</td>
<td>71.8</td>
<td>27.4</td>
<td>73.4</td>
<td>42.5</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>6.3</td>
<td>71.5</td>
<td>27.1</td>
<td>74.5</td>
<td>84.3</td>
<td>111.4</td>
</tr>
</tbody>
</table>

T<sub>i</sub>: Initial temperature  
T<sub>Paste</sub>: Temperature after pasteurization (72°C, 15 s)  
T<sub>TS</sub>: Temperature after thermosonication  
n/a: Not applicable  
<sup>*</sup>Note: 100 ml of skim milk = 0.104 kg; C<sub>p</sub> = 4.0 kJ/ kg°C

3.3.3. Total Plasmin Assay

A new centrifuge tube of milk was opened for each analysis. Total plasmin activity is defined as the activity of inactive plasminogen being converted to active plasmin in milk or cream samples [21]. The procedure for measuring the total plasmin activity is based on the methodology used by Politis et al. [19], which is a modification of their conventional methodology based on Rollema et al. [20]. An aliquot of 5 ml of skim milk sample was ultracentrifuged using a Beckman L8-M Ultracentrifuge (Bessey Hall, Iowa State University, Ames, IA) at 100,000 × g for 1 h at 4°C to separate the supernatant (milk serum) and the pellet (casein) fractions. This process ensured that plasmin and plasminogen remained intact in the casein micelles. The milk serum was removed from the casein and 50 mM of Tris buffer (pH 8.0) containing 110 mM NaCl and 50 mM ε-aminocaproic acid (EACA) was added to reconstitute to the original volume. The casein pellet was then resuspended in the
buffer solution and incubated at room temperature (20°C ± 3°C) for 2 h to ensure plasmin and plasminogen dissociated from casein and transferred to the buffer. Following incubation, the casein micelles solution was centrifuged again at 100,000 × g for 1 h at 4°C to allow the transfer of plasmin and plasminogen into the buffer.

For the absorbance analysis, 50 µl of supernatant buffer containing transferred plasmin and plasminogen was mixed with 950 µl of 50 mM of Trizma buffer (pH 7.4) containing 110 mM NaCl, 0.6 mM H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride and 2.5 mM EACA. Another 150 plough units of urokinase were added to this solution to convert inactive plasminogen to active plasmin. The reaction mixture was incubated for 60 min at 37°C for the sufficient conversion of plasminogen to plasmin [21]. The absorbance of conversion at 405 nm was measured using a Spectronic Genesys 2 UV-VIS Spectrophotometer (Scientific Support, Inc., Hayward, CA) at 30 min intervals for 3 hours. A similar reaction mixture without the milk serum fraction served as the blank. Absorbances were averaged for three replications, plotted as a function of time, and the rate of absorbance increase (p-nitroanilide formation) was calculated. The rates of p-nitroanilide formation were determined by calculating the regression of plasmin activity over time. From the slopes of the regression lines, the percentage of plasmin activity after treatment (heat and thermosonication) was calculated in Eq. (3.1), as follows:

\[
\%_{\text{plas. activity}} = 100 \frac{s_{\text{treated sample}}}{s_{\text{control}}} \quad (3.1)
\]

where \(s_{\text{treated sample}}\) is the slope of treated sample and \(s_{\text{control}}\) is the slope of control.
3.4. Results and Discussion

The total plasmin activity of skim milk were determined at bi-weekly intervals. The average plasmin activity (%) of skim milk over the time studied is seen in Figure 3.2.

Figure 3.2 Effect of thermal treatments and thermosonication on total plasmin activity (%) in skim milk. A-D Means with the same letter within a sample day (7, 21, 35 and 49) do not significantly differ (P < 0.05)

The results indicated that both thermal and thermosonication treatments decreased total plasmin activity. However, thermosonication was more effective in keeping the plasmin activity at a lower level over 49 days of storage than thermal treatments of skim milk, and these results support the work by Vijayakumar et al. [18], who reported that thermosonication at 133 and 152 µm from 1 to 3 min maintained lower residual of plasmin activity than raw milk. Because the amplitude was kept constant while the sonication times were varied, the highest thermosonication treatment of 170 µm for 60 s resulted in a significant decrease in plasmin activity compared to raw milk, thermal treatment, and
thermosonication at 170 µm for 10 s. However, there was no significant difference in the reduction of plasmin activity between thermosonication at 170 µm for 30 s and 170 µm for 60 s. Thermal treatment at 72°C for 15 s yielded a reduction of plasmin activity ranging between 24 and 37% over 49 days of storage.

In contrast, the total plasmin activity of heat-treated skim milk showed a significant increase in plasmin activity over storage of 49 days. This increase in plasmin activity can be attributed to the thermal inactivation of plasminogen activator inhibitor. Prado et al. [22] previously reported an 80% reduction in PAI and inactivation of PI by 36% when milk was heated to 74.5 °C for 15 s. The same trend has also been observed in higher plasmin activity along with decreased plasminogen level in pasteurized milk stored at 37°C for 80 h [23]. The inhibitors present in fresh milk are heat sensitive and are inactivated during thermal treatment. In contrast, the activators are known to be heat stable; thus, increased plasmin activity is observed upon storage in pasteurized milk [8, 23]. Another factor that likely contributed to the increase in plasmin activity in pasteurized skim milk samples is the refolding/renaturation of inactive enzymes to their original active state. Study has shown that the activity of plasmin and plasminogen was fully restored after enzymes were incubated at 65°C for 10 min and cooled to 37°C. It is postulated that high conformational stability and high resistance towards destructive reactions of unfolded plasmin allows the enzyme to refold upon cooling without losing its activity [24].

The irreversible inactivation of plasmin also showed observed no changes in p-nitroanilide formation in milk that underwent pasteurization above 65°C using similar chromogenic substance (V7127) at pH 7.4 [24]. Our results are also in agreement with another study which reported that low pasteurizations (60 to 70 °C), closer to conventional
pasteurization process temperatures (72°C, 15 s), did not significantly increase the plasmin activity compared to control raw milk even though studies reported the loss of heat sensitive plasmin inhibitors and plasminogen activator inhibitor [8, 12]. Measurement of residual plasmin activity after long pasteurizations indicated that plasmin and plasminogen have to be heated to 90 to 95°C for up to 3 min in order to achieve 90% inactivation [12]. However, the same amount of plasmin and plasminogen inactivation can be achieved with thermosonation at 170 µm for 60 s, 72°C, according to our study.

The postulated theory of using ultrasound to increase the rate of inactivation of enzymes as a result of native structure unfolding from thermal denaturation was tested [24]. This study reported a synergistic reaction of ultrasonic cavitation and thermal effect along with pasteurization to reduce plasmin and plasminogen activity. Our study tested the inactivation of milk spoilage enzyme plasmin at shorter times (< 60s) that are relevant to be integrated in the dairy industry. Thermosonation at 72°C, 170 µm (140 W) for three different times (10, 30, and 60 s) resulted in approximately 50 to 96% decrease in plasmin activity. Th The results support previous study of milk thermosonicated at different amplitudes (133 and 152 µm) and longer times (1 and 3 min) [18]. In addition, thermosonation of skim milk at 150 W at 61°C showed a decrease in activity of alkaline phosphatase by 44%, while conventional thermization reduced the activity by approximately 24%. Over the 49 days of storage at 4 ± 1°C the plasmin activity of thermosonicated samples did not show a significant increase in activity over time but all the thermosonicated skim milk activity values were less than the initial activity of raw milk. This effect may be attributed to the denaturation by sonication of the unfolded enzymes (after pasteurization) does not allow the refolding of the enzyme.
3.5. Conclusion

Investigation of short duration (≤ 60 s) batch thermosonication in terms of effectiveness of reducing plasmin activity revealed that thermosonication at 170 µm_{(p-p)} for 30 and 60 s, decreased the total plasmin activity by 83 and 96%, respectively for up to 49 days. Thermosonication is deemed appropriate as a method to extend the milk shelf life but further studies need to be done in scaled-up systems to test the feasibility of incorporating the technology in the industry.
3.6. References


CHAPTER 4
IMPACT OF CONTINUOUS PSYCHROSONICATION AT INDUSTRIALLY RELEVANT FLOW RATES ON PLASMIN ACTIVITY IN STORED WHOLE MILK

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‡Department of Food Science and Human Nutrition

Iowa State University

4.1. Abstract

The effect of psychrosonication in a continuous-flow ultrasonic system, operating at a frequency of 20 kHz, followed by batch pasteurization (62.8°C, 30 min), was studied in whole milk. Fresh raw milk was pumped from a pressurized holding tank at 4 and 6 L/min and through an ultrasonic reactor at constant amplitude of 12 µm peak-to-peak (p-p). The reactor was equipped with a donut-shaped horn. After psychrosonication, milk was either refrigerated or treated thermally by vat pasteurization (62.8°C, 30 min) then refrigerated (4°C). The total plasmin activity in stored whole milk was analyzed on days 1, 14, 28, and 35 and compared with control groups (raw and pasteurized milk). Neither psychrosonication alone nor psychrosonication with pasteurization reduced plasmin activity better than pasteurization alone. Continuous psychrosonication may not be an appropriate adjunct to pasteurization to extend milk shelf life.
4.2. Introduction

Ultrasound is defined as acoustic waves at frequencies above the human hearing limit (> 20 kHz) [1]. Over the last decade, ultrasound applications have progressed from being laboratory prototypes to use for research and diagnostics into a complete operational technology used commercially in industries across Europe and the United States. Sonication application has been further enhanced by high power ultrasound technology, especially in food processing [2]. High power sonication is known to have a major impact on reducing the rate of processes, consuming only a fraction of time and energy than normally needed for conventional process, with high reproducibility.

When power ultrasound is applied in a fluid medium, the sound waves propagate in alternate compression and rarefaction pressure regions, creating cavitation. Negative pressure during the expansion cycles causes the formation of small vapor-filled voids in the liquid, which is known as cavitation bubbles. During this non-linear acoustical vibration, a transient bubble increases its radius progressively beyond its equilibrium radius before collapsing drastically. Consequently, a high amount of energy with localized high temperature and pressure are released to assist chemical reactions [3].

This technology has been used in a wide range of chemical, biological, medical, and industrial fields for various applications. Research by Manas et al. [4], Raviyan et al. [5], and Villamel and DeJong [6] demonstrated inactivation of enzymes in food systems using heat and high power ultrasound. In addition, we have shown that thermosonication (72°C, 152 µm<sub>p-p</sub> for 3 min) can inactivate microorganisms and proteases without producing off-flavors in milk [7]. From these studies, thermosonication exhibits the potential to be an adjunct to pasteurization to extend milk shelf life by inactivating the proteases in milk.
Proteases are enzymes that depolymerize proteins to peptides. Plasmin (EC 3.4.21.7) is the principal indigenous proteinase found in milk together with from cathepsins and elastase. The plasmin system is composed of plasmin, its zymogen (plasminogen), plasmin inhibitors, plasminogen activators and plasminogen activator inhibitors. Plasmin undergoes proteolysis or depolymerization of protein that results in age gelation and off-flavor in stored milk. This process can be attributed to both native proteases and to proteases of psychrotrophs that survive pasteurization and grow during refrigerated storage. At a temperature of 20°C, psychrotrophs, especially *Pseudomonas*, produce twice the amount of proteases and lipase than at a temperature of 5°C In addition to being able to reproduce at a wide range of temperatures (5°C to 45°C), these proteases are highly heat stable and can withstand UHT pasteurization (140°C for 4 s). After surviving extreme heat treatments, proteases from psychrotrophs hydrolyze the casein proteins, mainly α-, β-, and κ-casein. The breakdown of κ-casein results in bitterness of the milk [8 - 11].

Short duration (≤ 60 s) thermosonication is more effective compared to pasteurization alone in reducing plasmin activity but it is not effective in reducing the total aerobic counts (TAC) at the same processing conditions [12]. Benner et al. [12] compared thermosonication and psychrosonication in batch systems and proposed that psychrosonication with subsequent heating may be an appropriate method to reduce the TAC and extend milk shelf life beyond that after HTST pasteurization. Thus, the present work investigates the plasmin activity in milk after psychrosonication alone and after psychrosonication with pasteurization.
4.3. Materials and Methods

4.3.1. Milk Preparation

Fresh raw whole milk was collected from the bulk tank of Iowa State University’s Dairy Farm (Ames, IA). Raw whole milk was transported to the Iowa State University Center for Crop Utilization Research (CCUR) pilot plant and the milk was refrigerated at 4°C for 1 h before treatment.

4.3.2. Ultrasonic Continuous Experiment

Two sets of control samples were prepared. Raw whole milk was stored in centrifuge tubes as control raw samples. For control pasteurized samples, 1000 ml of raw whole milk was heated in a covered double boiler stainless steel pot set on a hot plat. The milk was heated to 62.5°C and held there for 30 min, mimicking vat pasteurization. The milk was stirred frequently using a sanitized spatula to prevent foam and scum formation. Heated milk samples were stored in centrifuge tubes.

The continuous experiment for psychrosonication (PS) was conducted using a Branson 2000 Series bench-scale ultrasonic power unit (Branson Ultrasonics, Danbury, CT) capable of operating at 3.3 kW and 20 kHz as illustrated in Fig. 4.1. Approximately 30 L of milk (4 ± 2 °C) was pumped from a 38 L stainless steel ASME pressure tank with agitator (Graco Inc., Minneapolis, MN) set at 1 atm to an ultrasonic reactor where the Branson Ultrasonics “donut” horn (Sonico, Birmingham, United Kingdom) was installed (Figure 4.2). Prior to the addition of milk, the entire system was scrubbed with Oasis® Enforce Self-Foaming Chlorinated Alkaline Cleaner (Ecolab, St. Paul, MN), followed by Mikroklene® DF Iodine Based Detergent Sanitizer (Ecolab, St. Paul, MN). The volumetric flow rates were adjusted to 4 L/min and 6 L/min. The flow rates were chosen by choosing energy densities
(47.1 and 70.7 kJ/L) closest to the ones that gave the highest reduction in plasmin activity after thermosonication in the batch system (Chapter 3).

**Figure 4.1** Continuous flow experimental set-up.

**Figure 4.2** Branson ultrasonics “donut” shaped horn. (Picture by David Grewell)

The donut horn was designed for continuous flow operation. During sonication, the horn vibrates in a radial direction. The horn was placed in a vertical position inside the sonication chamber where most of the milk passed through the center of the horn. The experiment was repeated four times over the course of 12 weeks using new milk samples for each experiment.
Even though it was rather challenging to compare the two systems based on their type of operation, energy density was the parameter selected to compare their efficiencies [13]. A constant ultrasonic amplitude of 12 $\mu$m$_{p-p}$ at inner diameter was used for the donut horn. The flow rates were varied by adjusting the valve that was located between the sonication chamber and the sample outlet. The flow rates were sufficient to ensure contact with the horn for several seconds. However, it is important to note that the effect of reaction chamber design are not considered in these experiments. The sonication chamber was coated with TempCoat® (Impreglon, Inc., Baltimore, MD), a wear-resistant slide fluoropolymer coating with nonstick properties that has FDA approval.

Table 4.1. Psychrosonication treatment settings, average temperature before and after treatment, and average energy density.

<table>
<thead>
<tr>
<th></th>
<th>Amplitude (µm)</th>
<th>Pressure (atm)</th>
<th>Flow rate (L/min)</th>
<th>$T_0$ (°C)</th>
<th>$T_{PS}$ (°C)</th>
<th>$T_{Past}$ (°C)</th>
<th>Mean Energy Density of US (J/ml)$^i$</th>
<th>Mean Energy Density of past. (J/ml)$^ii$</th>
<th>Total Energy Density (J/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Raw</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>4.6</td>
<td>n/a</td>
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<td>Control Past</td>
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<td>n/a</td>
<td>n/a</td>
<td>5.4</td>
<td>n/a</td>
<td>63.5</td>
<td>n/a</td>
<td>23.6</td>
<td>23.6</td>
</tr>
<tr>
<td>Psychrosonication</td>
<td>12</td>
<td>1</td>
<td>4</td>
<td>5.6</td>
<td>16.3</td>
<td>63.3</td>
<td>70.7</td>
<td>19.1</td>
<td>89.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>6.2</td>
<td>18.5</td>
<td>63.3</td>
<td>47.1</td>
<td>18.2</td>
<td>65.3</td>
</tr>
</tbody>
</table>

$T_0$: Initial temperature  
$T_{Past}$: Temperature after pasteurization (63°C, 30 min)  
$T_{PS}$: Temperature after psychrosonication  
n/a: Not applicable  
$i$: Assumption: Ultrasonics is 70% efficient  
$ii$: Note: 100 ml of whole milk = 0.108 kg; $C_p = 3.77 \text{ kJ/kg}^\circ\text{C}$
4.3.3. Ultrasonic Relative Net Energy Gain

The total energy delivered into the milk ($E_{in}$) in batch thermosonication and continuous flow psychrosonication is described by Equations (4.1) and (4.2), respectively.

$$E_{in} = \frac{P_{avg} \cdot t}{V} \quad (4.1)$$

$$E_{in} = \frac{P_{avg}}{Q} \quad (4.2)$$

where $t$ is sonication time (s); $V$ is volume (L); $Q$ is volumetric flowrate (L/s); $P_{avg}$ is average power (W); $E_{in}$ is energy in and out (J/L).

The average power of the ultrasound ($P_{avg}$) was measured from the output of the power supply of the ultrasonic unit that combines the voltage, current and phase of the power received from electrical power to the converter, then averaged to obtain the average power dissipated into the medium [13]. For batch sonication (Chapter 3), the energy density ($E_{in}$) was calculated based on Equation (4.2); dividing the average energy (J) by volume of the milk that was used for the experiment (L). For the continuous system, energy density ($E_{in}$) was calculated by dividing the average energy by the volumetric flow rate ($Q$) as detailed in Equation (4.2).

4.3.4. Total Plasmin Assay

A new bottle of milk was opened for each analysis. Total plasmin activity is defined as the activity converting inactive plasminogen to active plasmin in milk or cream samples [16]. The procedure for measuring the total plasmin activity was based on the methodology used by Politis et al. [14], which was a modification of their conventional methodology based on Rollema et al. [15], as previously described in Chapter 3.
4.4. Results and Discussion

The plasmin activity of samples of whole milk raw (control group #1), pasteurized (control group #2), PS 4 L/min, PS 4 L/min & pasteurized, PS 6 L/min, and PS 6 L/min & pasteurized was determined at bi-weekly intervals. The average plasmin activity (%) of whole milk is seen in Figure 4.3.

![Figure 4.3](image)

**Figure 4.3** Effect of thermal treatments and psychrosonication on total plasmin activity (%) in skim milk. A–C Means with the same letter within a sample day (1, 14, 28 and 42) do not significantly differ (P < 0.05)

Raw samples and samples psychrosonicated at 4 and 6 L/min had similar average total plasmin activity throughout the storage period. In addition, pasteurized control samples and samples psychrosonicated at flow rates of 4 and 6 L/min with subsequent pasteurization also showed no significant differences in their total plasmin activity up to 28 days, but these thermally treated samples had significantly lower plasmin activity compared to the ones without pasteurization. This shows that thermal treatment reduced plasmin activity and
maintained it at lower level over 28 days of storage than in milk that was treated only with psychrosonication.

On day 42, there were no significant differences between the controls and the samples that were treated with psychrosonication and/or pasteurization. The plasmin activity values were not significantly lower than those of the initial plasmin activity of raw milk. Thermal treatment at 63°C for 30 min yielded a reduction of 38 to 46% in plasmin. This result was in agreement with study that reported a reduction of enzymes (alkaline phosphatase, γ-glutamyltranspeptidase, lactoperoxidase) activity in milk by 24% after heating to 61°C for 56.3 s. Our previous study showed an approximate reduction of 24 to 50% in total plasmin activity [7]. In addition, irreversible inactivation of milk plasmin activity is observed when heated from 65 to 92°C [17]. Skim milk that was treated with heat in previous experiment (Chapter 3) also showed an approximate reduction of 24 to 37% through 49 days of storage.

Milk that was treated with PS at 4 and 6 L/min, followed by pasteurization exhibited a plasmin activity reduction of 53 and 60%, respectively. In contrast, the plasmin activity of both samples that had been treated with PS only at 4 and 6 L/min exhibited increased activity by 14% on day 14, which differs significantly from the plasmin activity of PS and pasteurized samples. No literature is available on the effect of PS (4 to 6°C) on plasmin and plasminogen activity. However, zero inactivation of native milk enzymes was found after continuous sonication combined with heat at 55°C for 40.2 s. Additionally, when skim milk was sonicated without any pasteurization at 23.5°C, γ-glutamyltranspeptidase (GGTP) and lactoperoxidase (LPO) were only inactivated by 22% and 14%, respectively [6]. The trend of temperature dependence on lemon pectinesterase (PE) inactivation showed that continuous sonication from 40 to 60°C (35 µm, 200 W) had no significant inactivation effect on the
enzyme [18]. Thus, it can be postulated that the PS milk at 4 and 6 L/min at 4°C did not exhibit any significant reduction in enzyme activity compared to raw milk and our data from statistical analysis supports this theory.

4.5. Conclusions

The results of this study clearly demonstrated that psychrosonication alone (at 4 and 6 L/min, with energy densities of 47.1 and 70.7 kJ/L, respectively) did not reduce plasmin activity at a level higher than pasteurization of milk. PS followed by pasteurization reduced plasmin activity better than PS alone, but the factor that made the difference is the pasteurization and not the psychrosonication. In order to have a synergetic effect of plasmin inactivation using ultrasound, milk needs to be sonicated at temperatures between 60 to 92°C. In short, psychrosonication is not a feasible technology to extend milk shelf life.
4.6. References


CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Demands from consumers for fresh food with high quality sensory properties are on the rise, along with requirements for safety and substantial shelf life. Despite their high perishability, milk products need to compete against shelf stable beverages in the marketplace. Thus, fresh milk requires pasteurization to eliminate pathogens to safe levels and to decrease the rate of deterioration. The shelf life of milk is limited by heat stable proteases and microorganisms. There are many emerging technologies such as high pressure processing, pulsed electric fields, ultraviolet light treatment, and ultrasonication.

In the present work we demonstrated that batch thermosonication reduced plasmin activity by 83 to 96% for up to day 49 compared to control raw skim milk when sonicated at 170 μm(p-p) (140 W), at 72°C for 30 and 60 s, respectively. Despite the fact that non-active plasmin activity is able to return to its active native state after pasteurization as a consequence of enzyme refolding, thermosonication is more effective in maintaining lower residual plasmin activity compared to raw and pasteurized milk over 49 days.

In order to test the feasibility of sonication in a large-scale process, scale-up is considered an important aspect. Taking into consideration that a lower total aerobic bacterial count (TAC) is achieved by psychrosonication with subsequent pasteurization instead of thermosonication, the scale-up process was focused on psychrosonication. Continuous flow psychro-ultrasonication using a “donut” horn, used in large-scale raw milk treatment, was studied for potential scale-up. The flow rates for the process were set based on the energy density levels that yielded the most reduction rate from the batch system. The results of this
study showed that psychrosonication at 4°C alone at 4 and 6 L/min, with energy densities of 47.1 and 70.7 kJ/L, did not reduce the plasmin activity to values lower than those for raw milk. In contrast, the milk that was psychrosonicated then pasteurized showed lower plasmin activity than raw milk. No significant difference was found in plasmin activity between control pasteurized and psychrosonicated samples followed by pasteurization, thus confirming that the major reduction of plasmin activity was a result of pasteurization. Thus, psychrosonication is not a feasible technology to be used in the dairy industry to extend milk shelf life.

Future work should include:

1. Testing the enzyme kinetics for both thermosonication and psychrosonication with pasteurization using skim and whole milk for finding the optimum temperature-time-amplitude combination to inactivate plasmin and plasminogen.

2. Food grade, sterile equipment is also needed to for the continuous sonication chamber to eliminate cross contamination, as the presence of microorganisms may affect the total plasmin activity in the milk. An automatic system to set the flow rate in continuous system should be installed to increase the accuracy of the method when repeating the experiments.

3. Batch psychrosonication should also be conducted to study the effect on total plasmin activity so that the energy densities from the batch process can be used for scale-up. The storage time on psychrosonicated milk can also be increased before pasteurization to investigate the effectiveness of the method.
4. In addition, thermosonication needs to be tested also in scaled up processes in a sterile environment to better understand the process in a larger scale than batch process.
APPENDIX: EXPERIMENTAL DATA

Figure A.1 Plot of absorbance increase over 3 hours in thermonsonicated skim milk serum after 7 days of storage

Figure A.2 Plot of absorbance increase over 3 hours in thermonsonicated skim milk serum after 21 days of storage
Figure A.3 Plot of absorbance increase over 3 hours in thermosonicated skim milk serum after 35 days of storage

Figure A.4 Plot of absorbance increase over 3 hours in thermosonicated skim milk serum after 42 days of storage
Figure A.5 Plot of absorbance increase over 3 hours in psychrosonicated followed by pasteurization of whole milk serum after 1 day of storage.

Figure A.6 Plot of absorbance increase over 3 hours in psychrosonicated followed by pasteurization of whole milk serum after 14 day of storage.
Figure A.7 Plot of absorbance increase over 3 hours in psychrosonicated followed by pasteurization of whole milk serum after 28 day of storage

Figure A.8 Plot of absorbance increase over 3 hours in psychrosonicated followed by pasteurization of whole milk serum after 42 day of storage