Casein Micelles and their Properties: Polydispersity, Association with Vitamin A and Effect of Ultra-High Pressure Homogenization

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Casein Micelles and their Properties: Polydispersity, Association with Vitamin A and Effect of Ultra-High Pressure Homogenization

A Dissertation Presented for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Maneesh Sheenu Mohan
August 2014
DEDICATION

Dedicated to my grandfather for his ‘Never say die’ attitude.
I am grateful to Dr. Federico Harte for providing me the opportunity to pursue my PhD at UT in a subject of great interest to me. I greatly appreciate my committee members Dr. Qixin Zhong, Dr. Svetlana Zivanovic and Dr. Juan Jurat-Fuentes, for their constant cooperation and guidance. I appreciate all the help provided by Dr. John Dunlap for performing cryo-TEM, TEM and STEM. I would also like to thank Dr. Michael Davidson for keeping me motivated for the college bowl competition even towards the end of my PhD.

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Maneesha S. Mohan
ABSTRACT

The internal structure of casein micelles is not fully understood. In the present work, we explore some of the basic questions about casein micelles in bovine milk including its size distribution, native binding properties and effect of ultra-high pressure homogenization (ultra-HPH) on technological properties of casein micelles. The size distribution of casein micelles was studied by cryo-TEM using skim milk from four cows. The degree of variation in casein micelle sizes (polydispersity) ranged between 0.39 and 0.47 for a size distribution ranging from 10 to 693 nm. These results indicated high polydispersity of casein micelles in milk from single cows. Casein micelles associated with vitamin A in four pasteurized skim milks (1.6 - 2.5 micro gm/mL of milk; 14 - 40% of the initial quantity detected in milks), while other protein fractions contained negligible vitamin A. Thus, casein micelles can inherently associate with hydrophobic probes like vitamin A in milk. This association of vitamin A to casein micelles in milk provided protection from degradation on exposure to ultraviolet light when compared to apple juice. Further, the effect of ultra-HPH up to 500 MPa on the physicochemical (apparent casein micelle size by dynamic light scattering) and technological characteristics (rennet coagulation - firmness [90 min, 1Hz, 0.01% strain]; acid gelation using 3%w/v glucono delta lactone – firmness [22°C, 110 min, 1Hz, 0.01% strain], SDS-PAGE) of casein micelles were studied. Casein micelle size increased from ca. 180 nm at 100 MPa to ca. 280 nm at 500 MPa HPH pressure. With increase in HPH pressure, renneting ability decreased until no coagulation was obtained for 500 MPa HPH milk. The firmness of HPH milk acid gels increased from about 76 Pa to 108 Pa when pressure was increased above 100 MPa (up to 400 MPa) HPH as compared to acid gels made from non-homogenized milk. Overall we elucidated the size distribution, binding ability to
vitamin A and changes occurring on ultra-HPH in casein micelles. This information can be utilized by the industry to modify and utilize casein micelles as an ingredient for different end uses.
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW
Introduction

Bovine milk and milk products have been consumed as a source of nutrients and energy from prehistoric times. The first record of milk consumption by humans dates back to 9,000 years ago (Evershed et al., 2008). Cheese was first consumed 7,000 years ago (Salque et al., 2012). There are a variety of dairy products that are a staple in our modern human diet. Milk, mainly composed of water, carbohydrates, proteins and minerals, has been tailored by nature to transport these nutrients from the mother to the neonate (Farrell Jr. et al., 2004). Milk proteins include caseins and whey proteins. In the present dissertation, I will focus on casein proteins, their structure, binding properties and effect on high pressure homogenization.

Casein micelles: structure and function

Casein fractions

Caseins in milk comprise about 80% of the total milk proteins. The four major casein fractions are $\alpha_s1$, $\alpha_s2$, $\beta$ and $\kappa$ caseins in the proportion 4:1:3.5:1.5, respectively (Swaisgood, 2003). The $\alpha_s1$, $\alpha_s2$ and $\beta$ caseins are highly phosphorylated and $\kappa$-caseins are glycosylated. The $\alpha_s$, $\beta$ and $\kappa$ caseins are phosphorylated at specific serine residues and contain 8, 9-12, 5 and 1 phosphates respectively (Farrell Jr et al., 2004, Horne, 2006).

In $\alpha_s1$-casein, hydrophobic and charged residues are arranged close together in the protein sequence and causes clustering of the phosphorylated residues. The hydrophobic regions are roughly between 1-44, 90-113 and 132-199 (Swaisgood, 2003). The $\alpha_s2$-casein is the most hydrophilic among all the caseins with only two relatively hydrophobic regions 160-207 and 90-120. It has three clusters of phosphoseryl and glutamyl residues. The C terminal has a net positive charge at the pH of milk. There are two cysteine residues in $\alpha_s2$-casein that form intra-
molecular or inter-molecular disulphide bonds based on their monomeric or dimeric structures, respectively (Rasmussen et al., 1994).

β-casein is the most hydrophobic casein. It also has a highly charged N terminal domain with phosphoseryl clusters. The hydrophobic residues are found at the C terminal of the protein, making it amphipathic with a polar N terminal and hydrophobic C terminal. The primary structures of all the caseins and their genetic variants were given by Swaisgood (2003). About 60% of the total β-casein has been reported to leave and re-enter the micelle during temperature fluctuations (Creamer et al., 1977). This indicates that it is associated in the micelle to a large extent by hydrophobic interaction and to a lesser extent by ionic bridges (Downey and Murphy, 1970).

κ-casein is a glycosylated protein which is composed of carbohydrate moieties that are acetylneuramic acid, acetylgalactosamine, galactose and sialic acid (Doi et al., 1979, Tran and Baker, 1970). Trisaccharide or tetrasaccharide carbohydrate moieties attached to the 1 to 4 threonyl residues are also present in some κ-caseins (Fiat et al., 1972, Fournet et al., 1979, Tran and Baker, 1970). κ-casein can also form quaternary structures through intermolecular disulphide bonds (Swaisgood, 2003).

Caseins have a large number of proline residues that disrupt secondary structure formation including α-helix and β-sheets (Swaisgood, 2003). Hence caseins have relatively low secondary and tertiary structures and have different hydrophilic and hydrophobic regions. However, the existence of secondary structure of caseins is a question that has been debated extensively over the years. Some authors consider the caseins as rheomorphic or intrinsically disordered proteins and others as proteins forming molten globule structures (Horne, 2002). The essence of both
theories is that caseins are dynamic and adapt their conformation to specific environmental conditions (Holt and Sawyer, 1993). \( \alpha_{s1} \)-casein has a strongly acidic peptide of 40 amino acids that include seven phosphate groups, twelve carboxyl groups and four positively charged groups at native pH of milk (pH 6.6). While \( \beta \)-casein has an N terminal region containing five phosphates, seven carboxyl groups and two positively charged groups. The glycosylated \( \kappa \)-casein contains one phosphate group and fourteen carboxylic groups in the glycomacropeptide (hydrophilic terminal end region; Anal et al., 2008, Dalgleish, 1998). Much of the hydrophobic nature is associated with high charge, many prolines and the few cystine residues present in the caseins (Walstra, 1999). All these residues facilitate the existence of a number of forces among caseins including ionic bonds, electrostatic interactions, \( \text{vdW} \) forces, hydrogen bonds and hydrophobic interactions (Acharya et al., 2013, Livney, 2010). Some molecules have strong attraction to proline residues especially polyphenols (Maiti et al., 2007). Hence, they are intrinsically unstructured owing to the high net charge and hydrophobicity under native conditions (Dalgleish, 2011, Phadungath, 2005, Uversky, 2002). The elucidation of the tertiary structure of caseins is difficult as they cannot be crystallized for X ray crystallography and NMR studies owing to their very flexible tertiary structure (Alaimo et al., 1999). Caseins have also been described as block polymers based on their association among themselves (Horne, 1998). A block copolymer is defined as a macromolecule that constitutes of different types of units. However, the reason for the self-association of caseins, the forces and residues involved are still unknown.
**Micelle size**

Casein micelles from the same cow have been found to have varied size, that is, they are highly polydisperse. The casein micelle size distribution ranges from 80 to 600 nm in diameter, with an average size between 100 and 200 nm (Dalgleish, 2011, de Kruif, 1998, McMahon and Oommen, 2008, Schmidt et al., 1973, Schmidt et al., 1974). Apart from these large size micelles, a small population of mini-micelles with diameters between 20 and 60 nm were observed by light scattering and TEM techniques (Müller-Buschbaum et al., 2007). However, some groups have reported the smallest micelles to be around 50 nm (Udabage et al., 2003), while others consider it between 20 – 40 nm in diameter (Metwalli et al., 2008, Müller-Buschbaum et al., 2007). Thus, the minimum size of casein micelles is still disputed as different techniques are sensitive to different minima for size measurement. The casein micelles contain more than 20,000 individual proteins with molecular masses ranging from 19,000 to 23,000 Da and the particle mass ranging from $10^6$ to $10^7$ Da (Dalgleish, 2011, Farrell Jr et al., 2004, Holt et al., 2003). Researchers have over time agreed on a broad range for the size of casein micelles, although the use of different techniques is known to produce different size distributions (de Kruif, 1998, Martin et al., 2006).

The size of the casein micelles is correlated to the different casein fractions content and calcium phosphate ratio. κ-casein content is widely accepted as a determinant of micelle size (Dalgleish and Corredig, 2012). κ-casein content has been shown to decrease with increase in micelles size (Dalgleish et al., 1989, Marchin et al., 2007). The changes in micelle size have been reported to be consistent with colloidal calcium phosphate content in the micelles. Despite many studies on the correlation of αs and β caseins and micelle size, there is no consensus on whether these two
proteins play any role determining the final casein micelle size (Dalgleish et al., 1989, Marchin et al., 2007).

**Structure of casein micelles**

*Colloidal calcium phosphate nanoclusters.* The calcium sensitive caseins $\alpha_s$ and $\beta$ are highly phosphorylated. The phosphorylated serine residues form clusters of 2, 3, or 4 residues that bind calcium present in excess in the secretory vesicles in the mammary gland. About 65% of the 30 mM calcium in milk is encompassed in the nanoclusters (Holt, 1985, White and Davies, 1958). These nanoclusters are able to cross link the $\alpha_s$ and $\beta$ proteins by ionic bridges to form a three dimensional micelle structure (Horne, 2006). The diameter of nanoclusters range from 2 to 12 nm as observed by different research groups (de Kruif et al., 2012, Holt, 1998, McMahon and Oommen, 2008, Trejo et al., 2011). The nanoclusters are separated by an average distance of 18.6 nm (de Kruif et al., 2012), which is considered to be far away for the phosphoseryl residues of the same protein to bridge. Hence, many researchers have suggested the association between the casein to be noncovalent interactions among themselves (Dalgleish, 2011, Horne, 1998, Horne, 2006). These suggestions are based on calculation (Horne, 1998). The calcium phosphate nanocluster forms approximately 7% of the total dry mass of the casein micelles and there is an average of 700 to 800 nanoclusters in a $10^6$ kDa casein micelle (McMahon and Oommen, 2013, McMahon and Oommen, 2008, Trejo et al., 2011). Each nanocluster consists of 40 to 60 protein monomers (De Kruif and Holt, 2003, Holt, 2004, McMahon and Oommen, 2008). However, there is no actual information about the number of individual caseins associated within a nanocluster and their arrangement within the nanocluster.
Micelle structure. A number of studies were conducted on the casein micelle structure and many models were proposed (Dalgleish, 1998, Dalgleish, 2011, De Kruif and Holt, 2003, de Kruif, 1999, de Kruif et al., 2012, Fox and Brodkorb, 2008a, Horne, 2002, Horne, 2006, McMahon and Oommen, 2013, Walstra, 1999). However, the internal structure of the casein micelles still remains a subject of debate. There are few facts we know about the structure of the casein micelles.

The four fractions of caseins are considered to be homogenously distributed throughout the micelle without formation of any subunits (Dalgleish et al., 2004). Although, initially κ-casein was considered as the only proteins in the outer layer, latest studies using surface plasmon resonance based biosensor techniques have elucidated the presence of αs and β caseins together with κ-caseins on the surface of the casein micelles (Dupont et al., 2011). The amount of κ-casein present in casein micelles is thought to be insufficient to cover 39-48% of the surface area of the casein micelles (Dalgleish, 1998). Hence, there is no definite distinction of a κ-casein on the surface of the casein micelle, but rather a decrease in the density of proteins at the surface (Dalgleish et al., 2004). Although the outer layer contains all the caseins, the tail of the κ-casein extends out about 5-10 nm from the micelle surface (De Kruif and Zhulina, 1996, Horne, 1986). This tail part of the κ-casein molecules have close to 14 carboxylic acid groups that dissociate at milk pH (6.6) and promote solvation in the mineral-rich serum phase (de Kruif, 1999). Also, the κ-casein tails are far enough from each other to allow β-casein to leave and re-enter the micelle during temperature fluctuations (Creamer et al., 1977). It also allows whey proteins to form disulphide bonds with the inner part of the κ-casein (Anema and Li, 2003, Donato et al., 2007), and allows access for the proteolytic enzymes chymosin and trypsin (Diaz et al., 1996).
Despite remaining questions on the structure of the casein micelle outer layer, this region is fairly well resolved as compared to the internal structure of the caseins micelles. There is hardly any information about the arrangement of caseins within the structure of the micelles, apart from a number of predictions and models. The nanoclusters are distributed within the casein micelles randomly and hold together the \( \alpha \) and \( \beta \) caseins. Apart from this, the internal arrangement of proteins is still largely unresolved.

**Interactions or forces among caseins within micelles.** The integrity of the micelles has been associated with the ionic bridges formed by the phosphoseryl residues and calcium ions. However, even after dissolution of calcium phosphate by acidification to pH 4.9, the casein micelles maintained its integrity (Dalgleish and Law, 1989). All the internal forces responsible for holding the casein micelles together in this case were categorized as hydrogen and hydrophobic interactions (Horne, 2006, Horne, 1998, McGann and Fox, 1974).

The major force stabilizing the casein micelles from clumping together is the steric stabilization effect, induced by the protruding tail end of the \( \kappa \)-caseins on the surface of the casein micelles (Holt and Horne, 1996). It is explained based on free energy increase occurring in a condition of contact with another polymeric molecule. The micelles always prefer a more thermodynamically stable state, which is at lower free energy (de Kruif, 1999). Steric stabilization is a combination of repulsion of \( \kappa \)-caseins tails, electrostatic repulsion and van der Waals’ attractions (De Kruif and Zhulina, 1996, Tuinier and de Kruif, 2002). The equilibrium of these forces among casein micelles have been calculated by Tuinier and de Kruif (2002).

**Hydration.** The casein micelles are known to contain 3-4 kg water/kg of protein, which is split between the outer layer and the inner core of the micelle (De Kruif and Holt, 2003, de Kruif,
Out of all the water present, only 15% is considered to be bound water, the remainder is occluded within the micelle structure (De Kruif and Holt, 2003, Farrell Jr et al., 2003). Only a fraction of this water is thought to be present in the outer layer, which is rich in κ-casein (Dalgleish, 2011). The distribution of water within the casein micelles and the factors influencing this distribution are not clearly understood. The non-covalent interactions within the caseins micelles have long been labeled as hydrophobic interactions. However, the sole presence of hydrophobic interactions will eliminate any chances of the presence of water within the casein micelles (Dalgleish and Corredig, 2012, Dupont et al., 2011). Hence, a better understanding of the sites of hydration within the casein micelles needs to be attained.

The presence of channels and crevices within the casein micelles make it a highly porous structure. However, the biological function of these pores and channels is not yet understood. A number of researchers have attributed it to factors like association of occluded water within the micelles (Dalgleish, 2011, Dupont et al., 2011) and the ability of β-casein to move in and out of the micelle with temperature fluctuations (Creamer et al., 1977). The presence of large crevices and channels would also explain the uneven distribution of water within the micelle (Dalgleish, 2011).

**Existent models for the internal structure of the casein micelles.** The internal structure of the casein micelles has been studied by a number of research groups; however it is still being disputed. The different models proposed overtime include the submicelle (Walstra, 1990), dual binding (Horne, 1998), nanocluster (Holt et al., 2003), and Dalgleish’s (Dalgleish, 2011) models. All of these models are in consensus regarding the presence of κ-casein on the micelle surface and CCP clusters in the core of the micelle. In the submicelle model, αs and β caseins were
considered to form globular subparticles, which were coated or brought together by the calcium phosphate domains (Walstra, 1990). The presence of globular or distinct submicelles was, however, subsequently found to be artifacts of microscopy (McMahon and McManus, 1998). Results from small angle X-ray and neutron scattering studies indicated that structures exist within the micelle which causes a shoulder in scattering. These structures were later confirmed by several studies to be calcium phosphate and bound proteins forming nanoclusters dispersed throughout the micelle (Holt et al., 2003). This was the main basis for the nanocluster model which proposed the stabilization of micelles by nanoclusters and hydrophobic bonds. In the dual binding model which is analogous to the nanocluster model, Horne (1998) considered the interaction of phosphoserine clusters in the caseins with calcium phosphate and of hydrophobic amino acid residues as the main reason for polymerization of polyfunctional monomers. In the latest review of casein internal structure, Dalgleish (2011) postulated the role of water in the internal structure of casein micelles. He explained the incompatibility of the hydrophobic character of αs and β caseins in the proposed models with the highly hydrated structure of casein micelles. He suggested the presence of β-casein in a mobile form interacting with the water molecules due to its high amphiphillic nature. β-casein is considered to bind to some of the hydrophobic regions of calcium phosphate nanoclusters from the αs-caseins as well as act as a surfactant stabilizing the water channels in the casein micelle (Dalgleish, 2011).

Functions of casein micelles

Biological functions. The primary biological function of the casein micelles is as a transport system for nutrients from the mother to the neonate. The main nutritional components transported being calcium, phosphate and amino acids. The three dimensional structures of the
casein micelles enables incorporation of calcium and phosphate present in supersaturated levels in the mammary gland. This prevents the calcification and formation of stones within the mammary gland which can potentially block the milk duct (Fox and Brodkorb, 2008b). The structure of the casein micelles enable coagulation by the action of chymosin and formation of soft curd that is easily absorbed and digested in the neonatal stomach. This coagulation slows the entry of the milk to the small intestine and provides enough time for digestion and assimilation (Fox and Brodkorb, 2008b). Studies have also shown the ability of casein micelles to associate to hydrophobic compounds like fat soluble vitamins. It is not known whether such hydrophobic compounds are also loaded into the casein micelles during biosynthesis. A valid question is whether the physiological temperature of the cow enables the higher loading efficiency of these hydrophobic compounds into the casein micelles.

**Technological functions.** Casein micelles are highly heat stable and can be used in soups, gravies, and coffee creamers. Micelles coagulate at isoelectric pH of 4.6, which is beneficial in cultured milk products and desserts. Owing to its high level of hydration, casein micelles in their dehydrated form are used for water adsorption in bakery and cereal products. Caseins are highly surface active and are used as a film forming agent even in non-food applications like paper coating, leather coloring and glues (Adachi et al., 1976, Kuwabara et al., 2002). The chymosin activity in the calf stomach is effectively replicated in the production of cheese, where the protein matrix formed by the casein micelles entraps whey proteins and the serum phase. This makes cheese one of the most nutritious milk products. Recent studies have investigated the use of casein micelles as vehicles for the delivery of beneficial hydrophobic compounds like
triclosan, vitamin D$_2$ and Docosahexanoic acid (Roach et al., 2009, Semo et al., 2003, Zimet et al., 2011).

The ability of caseins to bind to hydrophobic compounds has been studied extensively and has been discussed in detail later in this chapter. However, there is no information about the residue or protein sequence that is responsible for this association and the type of forces and interactions that enable the association. The casein micelles have been shown to exhibit changes in its micellar size with application of high hydrostatic (0 to 600 MPa) and dynamic pressures (0 to 350 MPa), however the forces that play a role in these changes are still unknown. Many of the changes in micelle size have been associated with the changes in the hydrophobic interactions with change in temperature. Further details of the effect of high pressure on casein micelles have been discussed further on.

**Rheological properties of casein micelles**

The nano-rheology of the casein micelles has not been extensively studied. The few studies conducted using atomic force microscopy (assumptions are constant Poisson ratio of 0.5 and that the curvature of the casein micelle surface is much larger than the AFM tip) indicate the Young’s modulus to be ranging from 0.06 to 0.1 MPa (Helstad et al., 2005, Trejo et al., 2011, Uricanu et al., 2004). This value is similar for other soft biological materials, but higher than the elastic moduli of casein gels (Uricanu et al., 2004). These recorded values incorporate the effects of electrostatic repulsion (in between the casein micelle and the AFM tip), steric interactions ($\kappa$-casein and $\beta$-casein stabilization in the outer layer) and elasticity contributed by the load in contact with the chains of large number of proteins (Uricanu et al., 2004). The elasticity values were also found to be highly dependent on the temperatures and pH. Helstad et al. (2005)
interpreted the AFM results as caseins micelles having a hairy layer that behaves similar to a layer of casein adsorbed on to a hard hydrophobic surface and the inside of the micelle behaved like a linear spring.

The recent AFM studies have shown that the outside layer and the interior of the casein micelles are different in their rheology (Helstad et al., 2005, Uricanu et al., 2004). These studies have elucidated the average values for the elastic moduli of casein micelles. This still leaves a question of how different the elastic moduli of the outer layer and the interior of the casein micelles are. The reason for the lack of more studies on the nano-rheological properties is due to the very small size of the casein micelles. The highly deformable structure of the casein micelles also is defined as the reason for the immense amount of variability in the studies of size distributions and structure of casein micelles. The different techniques employed exert different types and magnitudes of forces and subject the micelles to different environmental conditions, which invariably affects the characteristics and functions of the casein micelles.

**Binding of casein micelles with hydrophobic probes**

*Caseins as nanocarriers*

There are many aspects about the casein micelles that are directly related to their interactions with other molecules. These interactions provide them the ability to act as nanocarriers of different beneficial moieties, especially important from the perspective of casein micelles as a vehicle for transporting calcium and phosphate to the neonate. In the present discussion we will be concentrating mainly on the ability of casein micelles to interact or bind with hydrophobic probes.
Owing to the hydrophobic forces acting between caseins within a casein micelle, it is possible to entrap, associate different hydrophobic probes with casein micelles.

Re-assembled casein micelles with α, and β caseins, sodium caseinates and phosphocaseins have been extensively used to study the interactions of caseins with hydrophobic probes. β-casein is a highly amphiphillic calcium sensitive phosphoproteins that exhibit self-association under different conditions in aqueous solution to form micelles (Liu and Guo, 2007, Portnaya et al., 2008). The radius of gyration of β-casein molecule is 4.6 nm and 15 to 60 of them cluster to form micelles of size 7.3 to 13.5 nm. The critical micelles concentration ranges between 0.05 and 0.2% w/v based on the conditions provided (Shapira et al., 2010a). Hence β-casein micelles have been utilized by a number of studies as carriers of hydrophobic probes including vitamin D, vitamin A and sucrose esters (Efrat et al., 2007, Forrest et al., 2005, Shapira et al., 2010b). The amphiphillic structure of β-casein has been compared to an amphiphillic diblock copolymer, when used as a nano-delivery vehicle for hydrophobic moieties (Dauphas et al., 2005, Horne, 2002, Mikheeva et al., 2003). The hydrophobic interaction of β-casein in solution prevents its self-compaction and formation of globular structures. Such block copolymers are more effective than surfactant micelles in entrapping hydrophobic molecules and release them at a much slower rate (Zana, 2005). This has been considered a major advantage of using β-casein as a nano-carrier (Shapira et al., 2010b). Vitamin D₃ was found to interact with β-casein in the molar ratio 1.16 - 2.05 per mole protein based on the solution conditions (Forrest et al., 2005). A patent has also been issued for using β-casein for preparing nanoparticles for hydrophobic additives at pH as low as 2.0, based on a study using vitamin D (Bargarum et al., 2009, Danino et al., 2009).
Re-assembled casein micelles from sodium caseinate have also been used in a nanocarrier system for various molecules. The size of such micelles is controlled by the amount of κ-casein in the casein mixture and the ionic concentration especially calcium and phosphate ions. The κ-caseins (occupy most of the casein micelles surface), when added in higher concentrations were found to produce smaller casein micelles. These smaller micelles exhibited higher stability to dialysis, pressure, ethanol and heat treatments (Schmidt, 1979). Micelles size was also shown to reduce when initial calcium concentration was reduced (Knoop et al., 1979). These micelles were considered quite similar to the naturally formed ones and this is believed to simulate the formation of micelles in the Golgi system of the mammary gland (Knoop et al., 1979).

Apart from the characteristics of amino acid residues producing various interactions among caseins and other added molecules, the quaternary structure of the casein micelles also promotes the incorporation of molecules. This is promoted by the open non-homogeneous structure with a large number of channels and crevices present in casein micelles (Trejo et al., 2011), which can hold 3 to 4 g of water/g of protein (Dalgleish, 2011) as explained earlier. Moreover, as mentioned earlier β-casein present in casein micelles moves in and out of the casein micelles with fluctuations in temperature (Creamer et al., 1977). This provides more opportunities for hydrophobic probes to associate to casein micelles in the place of removed β-caseins.

**Modes of interaction facilitating delivery tasks**

Milk proteins are known to bind to different molecules and ions by a number of different forces. Many of these mechanisms have been reviewed in detail (Livney, 2010). A few forces that are involved in the association of caseins and casein micelles with other moieties are hydrophobic interactions, van der Waal’s attraction and hydrogen bonds. For example, caseinate (Semo et al.,
2003, Semo et al., 2007) and isolated β-casein (Forrest et al., 2005) bind to vitamin D by hydrophobic interactions and β-casein binds mitoxantrone drug by a combination of hydrophobic and ionic interactions (Shapira et al., 2010b). The binding capacities of milk proteins to 2-nonanone, a flavor compound, decrease in the order: whey protein isolate > sodium caseinate and BSA > β-Lg > α-La > αs1-casein > β-casein (Kühn et al., 2007). The surface activity properties of caseins are also extensively used for encapsulation and delivery tasks (Grigoriev and Miller, 2009, McClements et al., 2007) and have been reported to decrease in the order: β-casein > monodispersed casein micelles > BSA > α-La > αs-casein = κ-casein > β-Lg (Singh and Ye, 2009).

Another mechanism is the co-assembly of hydrophobic probes with caseins during micelles formation owing to hydrophobic interactions (Semo et al., 2007, Sugiarto et al., 2009). Researchers employed co-assembly mechanism for loading casein micelles with hydrophobic drugs using a combination of ethanol and high pressure homogenization (Roach et al., 2009). Gelation has also been used as a cross-linking agent for entrapment of oral delivery protein drugs (Song et al., 2009). Heat-induced gelation produces sulphydryl bond between whey proteins and κ-casein and this has also been studied to enhance hydrophobic probe incorporation in to casein micelles (Yazdi and Corredig, 2011).

**Hydrophobic probes for binding with caseins and casein micelles**

Many studies have been conducted to study the hydrophobic binding of caseins and casein micelles. The utilization of casein micelles as a vehicle for hydrophobic probes has been investigated thoroughly in the past six years. One of the first studies conducted recovered 27% of added vitamin D_{2} from casein micelles on addition to skim milk. In the same study, about 58%
of the vitamin D$_2$ was obtained from the milk sera after ultracentrifugation, which was concluded to be associated with the soluble caseins in the study (Semo et al., 2007).

**Curcumin.** Curcumin, a plant derived lipid soluble compound has antioxidant, antitumorigenic and anti-inflammatory properties (Aggarwal et al., 2007, Joe et al., 2004). Curcumin was found to hydrophobically interact with non-polar regions of $\alpha_{s1}$-casein (at pH 7.4, 27°C) at two binding sites with varying affinities. Its antioxidant ability preventing hemolysis of erythrocytes was conserved despite its interaction with $\alpha_{s1}$-casein at 30°C (Sneharani et al., 2009). There are studies indicating hydrophobic interaction of curcumin with caseins in: casein micelle suspensions (obtained by re-suspending micelle pellet after ultracentrifugation (Sahu et al., 2008)), sodium caseinate suspension (Pan et al., 2013), phosphocaseins suspended in simulated milk ultrafiltrate (Benzaia et al., 2013), $\alpha_c$-casein and $\beta$-casein suspensions (Bourassa et al., 2013) and camel $\beta$-casein suspension (Esmaili et al., 2011). Diacetyl curcumin was also shown to associate by hydrophobic interactions to $\beta$-casein micelles at a single site in the core of the micelle with relatively high affinity (Mehranfar et al., 2013). The interaction with camel $\beta$-casein was found to increase the solubility, bioavailability and antioxidant activity of curcumin (Esmaili et al., 2011). The interaction of curcumin was different for $\alpha_c$-casein and $\beta$-casein with binding to 1.43 and 1.27 moles of curcumin, respectively (Bourassa et al., 2013).

**Polyphenols.** Another set of widely studied hydrophobic probes are tea polyphenols which include catechin (C), epicatechin (EC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG). Tea polyphenols have been known to interact with proline-rich proteins like salivary proteins and caseins (Fox, 2001). Green tea flavanoids prevented the binding of ANS to casein micelles surface by occupying the surface hydrophobic regions (Yuksel et al., 2010). The $\beta$-
sheet structure also decreased and the random coils and turns increased due to protein unfolding induced by polyphenol binding. The interaction between tea polyphenols and αs and β caseins reduced the α-helix structure in polyphenol-casein complex from 35-33% in free caseins to 32-24%. The interactions or binding of polyphenols increased with increase in the number of hydroxyl groups in the order of C ~ EC > EGC > EGCG in the same study (Hasni et al., 2011). The association of EGCG with caseins was found to reduce the renneting ability in micelles and non-micelles (Haratifar and Corredig, 2014). Glycosylated caseins obtained by maillard reaction were also found to encapsulate and retain EGCG without aggregation or fusion during storage (Xue et al., 2014).

Other plant polyphenols have also been studied in association with their interaction with caseins. Resveratrol is another plant polyphenol whose interactions with caseins have been studied. The number of resveratrol molecules binding with αs-casein and β-casein were 1.2 and 1.14, respectively (Bourassa et al., 2013). It also caused partial destabilization of secondary structure of caseins (Bourassa et al., 2013). It associated with sodium caseinate micelles mainly by hydrogen bonding and to a lesser extent by hydrophobic interactions (Acharya et al., 2013) and the association was spontaneous and exothermic. Genistein is another plant polyphenol with high antioxidative activity, which is found to bind to αs-casein and β-casein (Bourassa et al., 2013).

**Other probes.** Docosahexanoic acid is a fatty acid that binds at 3 to 4 sites of a protein molecule in reconstituted sodium caseinate micelles with and without addition of calcium and phosphate ions (Zimet et al., 2011). Some other drugs that have been shown to associate with β-casein micelles on being dispersed with using DMSO as solvent are mitoxantrone, vinblastine, irinotecan, paclitaxel and docetaxel (Shapira et al., 2010a, Shapira et al., 2010b). In these studies...
it was indicated that the mechanisms involved in the formation of such co-assembled nanoparticles depend on the charge and degree of hydrophobicity of the probes. The main forces that stabilize such structures were found to be hydrophobic and electrostatic interactions.

**Conditions affecting hydrophobic binding of casein micelles**

Despite the numerous studies on the association of caseins and its micelles, most of them have utilized \( \alpha_s \)-caseins, \( \beta \)-caseins, sodium caseinates or phosphocaseins. Very few of these studies have utilized the casein micelles found inherently in milk (Rahimi Yazdi et al., 2013, Sahu et al., 2008, Yazdi and Corredig, 2011). In many of these studies that use the native casein micelles, milk was ultracentrifuged to obtain casein micelle pellets, which were re-dispersed in milk ultrafiltrate, simulated milk ultrafiltrate (SMUF) or buffer solutions. Size exclusion chromatography is a much less disruptive process compared to ultracentrifugation at greater than 50,000 \( \times \) g.

**Effect of environment and processing conditions.** Another aspect is that all the reported studies indicate the ability of casein micelles to associate spontaneously with hydrophobic probes in an aqueous solution without any processing steps involved. There are a few studies on the effect of processing conditions like heating, pressure treatment and cooling on the binding of hydrophobic probes by caseins and casein micelles. Heating of proteins is known to induce conformational changes, exposing the hydrophobic sites (Bonomi et al., 1988, Kulmyrzaev et al., 2005). In milk, heat induced aggregation of whey proteins tend to produce complex formation with caseins (Donato and Guyomarc’h, 2009). The curcumin binding casein micelles heated at 80°C for 10 min was higher than in unheated milk caseins. The mechanism of binding was also slightly different in both samples. Curcumin penetrated the hydrophobic core of the unheated casein
micelles. The quenching and blue shift of the fluorescence intensity of tryptophan residues of caseins indicate their association with curcumin. Tryptophan has been associated with the hydrophobic association sites among caseins in the interior of the casein micelles. In the heated micelles the denatured whey proteins and aggregates increased binding with curcumin (Rahimi Yazdi and Corredig, 2012). Cooling affects the casein composition of micelles obtained by ultracentrifugation of skim milk and re-suspended milk ultrafiltrate. The β-caseins were released from the micelles on cooling (Creamer et al., 1977) and also the ionic concentration of supernatant increased (Rahimi-Yazdi et al., 2010). The release of about 60% of β-casein on cooling caused swelling of casein micelles by 12 ± 2 nm (Sood et al., 1997, Yazdi et al., 2014). This has been attributed to the loosening of structure with reduction of hydrophobic interactions and these sites were susceptible for occupation of curcumin (hydrophobic) and resveratrol (hydrophobic and hydrophilic; (Yazdi et al., 2014)).

**Effect of high pressure treatments.** A number of studies have investigated the effect of different types of pressure treatments including high hydrostatic pressure (HHP) and high pressure homogenization (HPH). HHP involves static high pressure and was found to increase the binding and loading capacity of curcumin (Yazdi et al., 2013) and vitamin D₂ (Menéndez-Aguirre et al., 2011) in casein micelles. The rearrangements occurring within the casein micelles on application of pressure (>200 MPa) favor binding to hydrophobic probes. Whey proteins, calcium and phosphate ions are also known to contribute to this high pressure effect.

High pressure homogenization on the other hand induces dynamic pressure involving other forces including cavitation, turbulence, shear stress and thermal energy (Paquin, 1999). Apart from the spontaneous binding of α-tocopherol acetate to phosphocasein dispersion in water (1:1
ratio), more binding was observed at HPH pressures ≥ 200 MPa. This was also obvious from the 1.5 times higher binding efficiency at 250 MPa and 3 times higher at 300 MPa compared to control without treatment (Chevalier-Lucia et al., 2011). This is attributed to the changes in micelle assembly during HPH by the dissociation and re-association to form smaller compact structures that spontaneously entrap hydrophobic molecules during its formation. Similar observations were reported by another group indicating a 1.5 to 2 fold increase in the binding efficiency of curcumin to native phosphocasein suspended in simulated milk ultrafiltrate (SMUF) at 300 MPa HPH pressure (Benzaria et al., 2013). Pressure below 200 MPa (Semo et al., 2007) and presence of small quantities of ethanol (Chevalier-Lucia et al., 2011) did not modify or change the size of the casein micelles. The increment of binding efficiency is due to the increase in hydrophobic interactions within the casein micelles. This is further proven by the increase in binding of α-tocopherol acetate to casein micelles at 34°C compared to that at 14°C. The hydrophobic interactions are known to strengthen at higher temperatures and this can play a role in the changes occurring in casein micelles during HPH which induces a short heating period.

Another aspect that has been scarcely explored is the storage stability of such nanocarriers formed by the association of different hydrophobic probes with caseins and its micelles. One study has indicated that rearrangements occur in casein micelles that result in a significant release of the bound curcumin (Rahimi Yazdi et al., 2013). Another group indicates a protective effect of reconstituted sodium caseinate micelles on vitamin D2 associated with it against ultraviolet light. This was attributed to the shade effect of casein micelles by which it absorbs and blocks ultraviolet light (Semo et al., 2007). Also there are a few studies indicating that
caseins and their micelles retain the biological activity of various hydrophobic probes associated with them (Esmaili et al., 2011, Mehranfar et al., 2013, Pan et al., 2013, Sahu et al., 2008, Sneharani et al., 2009). However, more studies are required to understand specifically the ability of casein micelles to protect the hydrophobic probes associated with them from degradation. In our research we have also made an effort to study the protection ability provided by casein micelles to vitamin A added to milk. This is especially important as vitamin A is commercially fortified in milk at a concentration of greater than 2000 IU.

**Effect of high pressure homogenization (HPH) on the physico-chemical properties of milk**

*Pressure treatment studies*
High pressure treatment emerged as a non-thermal processing technology suitable for food processing during the past three decades, owing to the need to replace thermal processing with a much more economical and less nutrient deteriorative process. High pressure emerged as an ideal option as it provided anti-microbial effects without changing the sensory and nutritive quality of foods. Pressure treatment is applied in food systems mainly by two methods, viz., high hydrostatic pressure (HHP) that applies hydrostatic pressure as high as 1000 MPa in a closed chamber, and high pressure homogenization (HPH), a continuous process with multiple forces (hydrostatic pressure, cavitation, shear stress, turbulence and thermal energy) acting in the system (Datta et al., 2005, Diels and Michiels, 2006, Donsì et al., 2009). Maximum pressure so far tried for HPH is 400 MPa.

*Principles of high pressure processing*
Pressure treatment of food affects its physicochemical, functional, and technological properties, which reflect the changes occurring in the constitutive biomolecules in the foods. When exposed
to pressure treatment, biomolecules follow the Le Chatelier-Braun principle, which states that when pressure is applied to a system in equilibrium, the system will counteract mainly by reducing the volume (Huppertz et al., 2002). Increase in pressure in a system is also associated with increase in temperatures especially in the case of HPH.

The effect of high pressure treatment on the proteins in foods has been of interest to many. The primary structure of food proteins consists of covalent bonds that are not generally affected by high pressure treatments unless accompanied with very high thermal treatment (Mozhaev et al., 1994). Secondary structure of proteins is mainly stabilized by hydrogen bonds that have been shown to be affected by pressure treatment. At low pressures (<150 MPa), hydrogen bonds are enhanced and they are disrupted at very high pressures (Hendrickx et al., 1998). High pressure treatments has been suggested as to destabilize electrostatic interactions between the charges in the vicinity of each ion caused by the decrease in volume of the water layers, associated with these charges or ions (Gross and Jaenicke, 1994). The hydrophobic and ionic interactions contribute to the tertiary structure of proteins. With the reduction in volume owing to pressure, hydrophobic interactions are also affected, changing the proximity of hydrophobic groups (Heremans, 1982, Mozhaev et al., 1994). Studies have shown that quaternary structure of proteins stabilized by non-covalent bonds are disrupted even at pressures >150 MPa. Hence, many multimeric proteins are broken down into monomeric proteins on exposure to high pressure treatments (Gross and Jaenicke, 1994, Hendrickx et al., 1998).

In this section, I will be discussing the effects of high pressure homogenization on milk components and milk system as a whole. A number of detailed reviews have been published on
the effects of HHP on the properties of milk and other food systems (Considine et al., 2007, Huppertz et al., 2002, López-Fandiño, 2006, Messens et al., 1997).

**High pressure homogenization equipment**

High pressure homogenization is a recent advancement in food processing, developed in the past 10 years. Most industrial pilot or lab-scale high pressure homogenizers have a plunger type pump and a valve. The commonly used types of valves are micro fluidics, ceramic needle and seat, ceramic ball and seat, and nozzle made from diamond, sapphire, or ruby. Another major component in an HPH system is the attenuator which is a constant volume chamber that stabilizes fluctuations in pressure. So far, the systems developed to study food systems can achieve maximum pressures of up to 400 MPa. However, outside the foods and pharmaceutical industry, a system has been developed using high pressure water for cutting very hard materials. Such systems, so called “water jets”, can achieve pressures as high as 600 MPa with the help of a nozzle made of diamond, ruby or sapphire having an orifice diameter less than 0.35 mm. This system has a high pressure piston pump connected to an attenuator that provides the back pressure in the system to achieve very high steady pressures.

**Effect of HPH on the properties of milk**

The thrust in the utilization of HPH in dairy industry is owing to its potential to continuously process milk or fluid dairy products without affecting nutritional quality and inducing maillard browning reactions. At the same time, this process exhibits the capability to inactivate microorganisms and enzymes. The added benefit to all of this is the possibility to selectively modify milk proteins and widen its scope of utilization as an ingredient with varied properties in the food industry.
**HPH induced temperature elevation.** A major attribute of HPH is the increase in temperature that influences a number of properties of milk proteins. As mentioned earlier, the increase in temperature is induced by different phenomena including shear stress, turbulence, cavitation, friction, and the transformation of kinetic energy to heat when the pressure increases in the system (Datta et al., 2005, Diels and Michiels, 2006, Donsì et al., 2009). The temperature increase is produced by adiabatic heating in addition to shear, cavitation and turbulence of fluid while exiting the homogenization valve (Hayes and Kelly, 2003).

A number of HPH studies have reported varying rates of heating on increasing pressure with or without temperature control before and after HPH. Fiejoo and coworkers reported a temperature increase of 19.3°C/100 MPa during micro fluidization of ice-cream mixes (12% fat) at pressures 50 to 200 MPa (Feijoo et al., 1997). The HPH of raw whole milk with inlet temperatures of 6 to 10°C increased the outlet temperature by 17.6°C/100 MPa on applying pressure in the range of 50 to 200 MPa (Hayes and Kelly, 2003). They also observed an increase in heating with increase in the fat content of the samples in a linear manner at the rate of 0.5°C for every 1% increase in fat content. This was explained by the increase in the population of particles in the form of fat globules that tend to collide with each other and exert more shear and other forces upon each other. Another group studied the elevation in outlet temperature with control of inlet temperature, obtaining a linear increase in rate of 0.6°C of outlet temperature with every 1°C rise of the inlet temperature (Datta et al., 2005). The increase in temperature during HPH can also be due to the number of passes employed along with the effect of increase in pressure (Sandra and Dalgleish, 2005). The control of temperature during the homogenization procedure reduced the rate of temperature with increase in pressure to a maximum of 10°C at 300 MPa (Roach and
The studies conducted more recently have established more temperature control in
the system with a temperature elevation rate of 14.8°C (Zamora et al., 2012b) and 16.6°C (Hayes
et al., 2005) per 100 MPa increase in pressure. A temperature increase of 45 to 60°C was
reported from the inlet point in to HPH to the outlet point (Amador-Espejo et al., 2014). In total,
the rate of temperature elevation remained in the range of 15 to 20°C for every 100 MPa increase
in pressure, regardless of differences in the inlet temperature and HPH equipment. Further, the
effect of increase in pressure, temperature and other forces on HPH will be reviewed in
association with the various properties of milk and milk products.

**Effect of HPH on the physico-chemical properties of milk**

**Particle size and structure.** The particle size of milk follows a bimodal distribution pattern with
separate peaks for fat globules and casein micelles. The casein micelle peak was reported to have
a maximum number of fractions at 0.2 μm and the fat globules at 3.7 μm (Amador-Espejo et al.,
2014, Zamora et al., 2012a). The bimodal distribution formed a monomodal pattern when
homogenized at 200 MPa with an increase in the 0.2 μm at an inlet temperature of 55°C. At 300
MPa, the bimodal distribution was retained with a reduction in the 0.2 μm peak and the pressure
of a tail region at the end of the 3.7 μm peak. The explanation for the observation of this tail
region was given based on the aggregation phenomenon. More fat globules are created with
HPH. These fat globules are stabilized by casein micelles at lower pressures ranging from 30 to
90 MPa, and at pressure greater than 200 MPa they become the limiting factor. The depletion of
casein micelles from fat globule surface at pressures >200 MPa HPH, causes partial
agglomeration of small fat globules to form the tail region in the size distribution (Amador-
Espejo et al., 2014, Hayes et al., 2005, Pereda et al., 2007, Thiebaud et al., 2003)
The size of the fat globules reduced considerably from 286 to 166 nm when HPH pressure was increased from 100 to 300 MPa (Zamora et al., 2012a). The same study indicated a decrease in fat globule diameter by 100, 60 and 20 nm, when the outlet temperature increased from 20 to 40°C for homogenization pressures 100, 200, and 300 MPa respectively. The fat globule size decreased until a HPH pressure of 200 MPa, while there was no further reduction at HPH pressure of 300 MPa of fat temperatures 30 and 40°C (18 MPa). These fat globule sizes were still bigger than those obtained from traditional homogenization processes (Pereda et al., 2007) and smaller than that from pasteurization process.

Casein micelle size in milk samples were found to be of the same size up to an HPH pressure of 150 MPa and decreased at 200 MPa HPH pressure (Hayes and Kelly, 2003, Sandra and Dalgleish, 2005). However, another study reported a decrease in particle size from 250 to 160 nm on increasing pressure from 0 to 200 MPa HPH pressure. On increasing the HPH pressure further, the casein micelles increased in size to about 200 nm (Roach and Harte, 2008). Although the pattern of reduction of casein micelle size is different as reported by different authors, this has been consistently attributed to the surface disruption of the casein micelles. This surface disruption theory was further strengthened by the observation of κ and αs casein in the serum phase of milk by gel electrophoresis (Roach and Harte, 2008, Sandra and Dalgleish, 2005). The increase in casein micelle size at HPH pressure greater than 200 MPa is probably due to the strengthening of hydrophobic interactions among caseins on exposure of hydrophobic regions to high pressures. The chances of complex formation of β-Lg with casein were eliminated as it was not precipitated with caseins (Roach and Harte, 2008).
Another factor that was shown to affect the casein micelle size in milk with HPH is the concentration of colloidal calcium and free calcium in the system. Roach and Harte (2008) reported an increase in the amount of free calcium ions in raw skim milk on increasing the HPH pressure from 0 to 300 MPa. The initial reduction in casein micelle size up to a pressure of 200 MPa and later on an increase in micelle size up to 300 MPa HPH was correlated with the free calcium ion concentration as well. Implicit in this observation is that below a particular level, colloidal calcium phosphate is lost from the casein micelles, which start to aggregate rapidly and increase in size. Calcium concentration is but one of the factors that affects casein micelle size. Overall, there are a number of other factors that needs to be closely monitored to understand the exact phenomenon causing changes in casein micelle size with application of HPH.

**Viscosity.** Viscosity is an essential physical parameter that determines the quality and sensory perception of a fluid. Changes in viscosity are correlated with changes in particle size, structure and interactions of the components in the fluid. In the case of milk, viscosity is mainly influenced by the particle size of casein micelles (Roach and Harte, 2008) and fat globules (Desrumaux and Marcand, 2002, Floury et al., 2000, Walstra and Jenness, 1984) as well as the denaturation of whey proteins.

Generally the viscosity of milk samples increases with increase in homogenization pressures. This has been demonstrated with an increase of 2.3 to 2.4 mPa.s for milk (3.5 % fat) on subjecting to HPH pressures of 200 and 300 MPa for inlet temperature 30°C, respectively (Pereda et al., 2007). Another group indicated an increase from 1.3 to 1.8 mPa.s for milk (3.5% fat) on subjecting to HPH pressures 200 and 300 MPa for inlet temperature of 55°C, respectively.
(Amador-Espejo et al., 2014). However, similar increase in viscosity was not observed for skim milks when homogenization pressure was increased from 100 to 300 MPa (Serra et al., 2008). Increase in viscosity in these studies has been associated with the formation of large aggregates that increase the volume of the dispersed phase (Amador-Espejo et al., 2014). The formation of such large aggregates has also been associated with denaturation of whey proteins with increase in temperature (Walstra et al., 2006), mostly above 70°C depending on the time period of exposure. High pressure homogenization is known to produce high temperatures. However, based on the type of homogenization treatment varying levels of denaturation of whey proteins have been observed by different researchers. Another major factor that affects viscosity of milk is the size of fat globules. Fat globules in whole milk are stabilized to some extent by casein micelles. Once the size of fat globules is broken down considerably with increase in homogenization pressures, these caseins act as the limiting factor inducing partial aggregation. This again increases the volume of the dispersed phase and viscosity. Aggregation of casein micelles has been related to the solubilization of colloidal calcium phosphate.

Inlet temperature and the number of passes are other factors that can affect the viscosity of milk. With increase in inlet temperature of milk (3.5 % protein content) from 55 to 85°C, viscosity increased from 1.8 to 2.4 mPa.s at 300 MPa homogenizing pressure (Amador-Espejo et al., 2014). However, a 10°C increase in inlet temperature from 30 to 40°C did not result in any significant change in viscosity (Pereda et al., 2007). The increase in inlet temperature of milk before homogenization, shown to increase viscosity, can in turn provide thermal energy in the system to enable whey protein denaturation. The utilization of a second stage of homogenization at a lower pressure (30 MPa) increases the viscosity of skim milk at 300 MPa homogenization.
pressure (Serra et al., 2008). The increase in viscosity has been attributed to the formation of protein aggregates (whey proteins and caseins) in the milk. This is due to the second stage of homogenization acting as a bottleneck and retarding the high velocity of the fluid, allowing the proteins to interact (Serra et al., 2007).

Whey protein has been indicated as a major reason for increase in viscosity on HPH of mixtures of sodium caseinate (2.5%) and whey proteins (1.25%) at 97 MPa for three passes (Venir et al., 2010). Whey proteins exhibited a synergistic relationship with sodium caseinate and increased the viscosity more than when these dispersions were used alone. The voluminosity of whey proteins as calculated using Lee’s equation from the relative viscosity indicated an increase with higher pressure from 100 (2.3 mL/g) to 300 MPa (2.5 mL/g) homogenization pressure on a 10% whey protein isolate dispersion (Grácia-Juliá et al., 2008). In many of these studies, the increase in viscosity was accompanied by a corresponding increase in particle size as well as fat globules and/or proteins structures (Amador-Espejo et al., 2014, Grácia-Juliá et al., 2008, Serra et al., 2008). Hence, viscosity measurement helps in further understanding the changes occurring in the complex milk system and the interactions associated with these phenomena.

**pH.** Most of the studies conducted for HPH indicated a lack of variation in the pH of homogenized milk (100 to 300 MPa pressures) samples. However, some decrease in pH has been reported on storage of HPH milks at refrigeration conditions. A decrease of 0.3 pH units on storage for 24 h and a reduction of 0.6 pH units on storage for 14 days have been reported (Hayes et al., 2005, Hayes and Kelly, 2003). The reduction in pH was accompanied by an increase in titrable acidity as well (Pereda et al., 2007). The reason for this reduction in pH has been associated with the presence of lipoprotein lipase (LPL) that destroys the fat globule
membrane, causing hydrolysis of fats due to the increased exposed surface area of fat globules after homogenization (Hayes and Kelly, 2003). However, most of these studies have not associated this pH reduction to the microbial concentration and production of lactic acid bacteria on storage. Hence more studies need be conducted to elucidate the factors affecting pH reduction with time in HPH milks.

**Ionic concentration.** The colloidal and ionic calcium phosphates in milk are important determinants of structure and size of casein micelles (Gaucheron, 2005). There are very few studies indicating the ionic concentration of HPH milks. On HPH, the calcium and phosphate ions increase at a homogenization pressure of 300 MPa (Amador-Espejo et al., 2014, Roach and Harte, 2008, Serra et al., 2008, Zamora et al., 2007). This increase in minerals in the soluble phase is correlated to the disruption of casein micelles owing to HPH and solubilization of colloidal casein phosphate. Although the trend observed was similar, the values of ion concentration reported were very different. One group reported soluble calcium levels in milk measured using a calcium combination electrode as increasing from 135 mg/L at 100 MPa to 160 mg/L at 300 MPa homogenization pressure (Roach and Harte, 2008), while the other reported calcium levels measured using inductively coupled plasma optical emission spectroscopy after diluting with nitric acid as increasing from 350 mg/L at 100 MPa to 450 mg/L at 300 MPa HPH in whey samples (Serra et al., 2008). This indicates the huge variability in mineral analysis data, although different techniques should provide similar results. The only reason for variability that can be elucidated is the differences in the processing conditions and temperatures used in different studies that affect the ionic concentration. Addition of 10 and 15 mM CaCl₂ buffer solutions to nano-titrated casein suspension increased the particle size of the casein micelles
significantly at 300 and 350 MPa HPH pressures and caused micellar precipitation on centrifugation. Apart from the effect of high pressure, the soluble ionic concentration of milk is also affected by thermal treatments. The solubility of minerals is greatly reduced at elevated temperatures. The increase in temperature during HPH, could also be an added factor for variations in the ionic concentration in milks subjected to different homogenization pressures.

**Denaturation of whey proteins.** Studies on denaturation of whey proteins are especially important as it affects many technological properties in dairy production including acid gelation. A better understanding of the interactions between the milk proteins will provide a better idea of the structural changes occurring during HPH in milk. Whey protein denaturation is commonly studied using SDS-PAGE. The two main whey proteins α-La and β-Lg are commonly involved in interactions among themselves and with casein on denaturation. They can form disulphide linkages or hydrophobic interactions among themselves or with caseins. They can exist as dimers, trimers, and aggregates owing to formation of disulphide linkages. A number of studies have been conducted on the degree of denaturation of whey proteins on varying thermal treatments (Claeys et al., 2001, Oldfield et al., 2000, Villamiel et al., 1997).

In early studies utilizing HPH up to 200 MPa pressure, no changes where observed in the secondary structure of the whey proteins (Hayes et al., 2005, Subirade et al., 1998). HPH was found to induce whey protein denaturation at higher pressure by other researchers. β-Lg was found to be more susceptible to denaturation than α-La (Hayes et al., 2005, Rüegg et al., 1977). The level of denaturation of β-Lg was 10, 42, and 56% for 150, 200, and 250 MPa homogenized milks, respectively (Hayes et al., 2005). About 32% of β-Lg denaturation was observed when pressure of 200 MPa was applied to whole milk while 47% denaturation was observed on
thermal treatment at 82°C for 8 min (Venir et al., 2010). An equivalent thermal treatment applied to a HPH pressure of 200 MPa was calculated to be 72 to 75°C for 15 seconds to one minute. Denaturation levels of 8 to 20% for temperatures 72.5 to 75°C for 15 seconds to one minute during thermal treatment indicated that the level of denaturation for HPH is very high (Oldfield et al., 2000, Villamiel et al., 1997). Hence most of the denaturation during HPH has been associated with the synergistic effects of the shear forces acting and the temperature (Datta et al., 2005). One of these studies evaluated the effects of HPH on a mixture of sodium caseinate and whey proteins (Venir et al., 2010). According to them, milk proteins are partially unfolded during HPH. This exposes junction zones within the proteins that enable the formation of intermolecular interactions rather than intra-molecular interactions forming an inter-penetrating polymer network that is not covalently bonded. However, it cannot be separated unless chemical bonds are broken (Work et al., 2004). All these studies indicate that denaturation of whey proteins occur on HPH, leading to their interactions with casein micelles, reforming the micelles. From other studies conducted on whey protein isolate solutions (6 and 10% w/v concentrations), it was concluded that whey protein aggregation occurs on HPH for pressures ≥ 250 MPa rather than whey protein denaturation. The aggregates formed were very small, i.e., less than 100 nm size (Grácia-Juliá et al., 2008). The aggregates were found to be soluble at pH 6.5 and lost its solubility (13 to 28% loss) at pH 4.7 above HPH pressures of 275 to 300 MPa. The amount of whey proteins (α-La, β-Lg and, BSA) as elucidated using gel electrophoresis indicated no changes in band intensity in the same study under reducing and non-reducing conditions. This observation was associated with the aggregates of whey proteins being dissociated by sodium dodecyl sulphate urea during SDS-PAGE. Therefore, it was concluded that the aggregates are
linked by hydrophobic interactions (Bouaouina et al., 2006). Overall, the contradictory studies on the absence and presence of whey protein denaturation on HPH above 200 MPa could be due to the variation in the types of systems used by the different research groups for HPH. The type of equipment, the inlet temperature, and the forces acting within the system are major factors that can affect the unfolding and denaturation of whey proteins.

**Ethanol stability.** Ethanol stability of milk subjected to high hydrostatic pressure has been extensively studied. However, there are hardly any studies on the ethanol stability of HPH milks. A lone study indicated that ethanol stability decreased with increase in HPH from 200 to 300 MPa (Amador-Espejo et al., 2014). This decrease in ethanol stability has been related to the increase in soluble calcium at higher HPH pressures and the possible removal of κ-casein from the surface of the casein micelle. The removal of κ-casein from the casein micelle surface tends to cause reduction of steric repulsion. The reduction in the level of calcium in the serum phase has also been reported for HPH milks (Zamora et al., 2007). The thermal energy produced during HPH could affect the amount of calcium in the soluble form as indicated by thermal treatment studies in the past. On application of heat treatments to milk, soluble calcium is converted into colloidal form and therefore affects the steric stability of the micelles (Fox and McSweeney, 1998, Horne and Muir, 1990). However, much needs to be understood about the combined effect of pressure and heat that has the potential to reduce the ethanol stability of HPH milks.

**Effect of HPH on technological properties of milk.**

**Effect of HPH on rennet coagulation and cheese characteristics.** The composition of milk plays an important role in the properties of the produced cheese. Other factors previously reported to affect cheese characteristics include thermal treatment, fat content, composition of
milk and homogenization. The mechanism of cheese curd formation involves destabilization of casein micelles by the action of chymosin enzyme on the κ-casein at the surface of the casein micelles (de Kruif, 1999, McMahon and Brown, 1984). The steric repulsion between micelle and the net negative charge of the micelles are reduced causing destabilization and later aggregation forming the three dimensional network (McMahon and Brown, 1984). Fat globules play a major role in cheese structure. They participate in the protein network formation and are entrapped within the network (Michalski et al., 2002). Hence, traditional homogenization (double stage 15 MPa and 3 MPa pressures) is known to affect cheese characteristics. The reduction of fat globule size, increases the moisture content and yield of cheese (Jana and Upadhyay, 1992, Peters, 1956).

Another factor that affects cheese structure is the incorporation of whey proteins. This is mainly affected by the thermal treatment the milk. Temperature above 65°C causes whey protein denaturation. These denatured whey proteins are partly or wholly bound to casein micelles (Anema and Li, 2003, Corredig and Dalgleish, 1999, Oldfield et al., 1998) and also can form soluble complexes and aggregates (Ménard et al., 2005, Mollé et al., 2006). On heat treatment at temperature >90°C for >10 min the rennet coagulation is negatively affected. This is due to complex formation of denatured whey proteins with κ-casein via disulphide bonds, which covers the surface of the casein micelles and hinders their interaction and aggregation to form network (Fox et al., 2000, Van Hooydonk et al., 1987, Vasbinder et al., 2003). Rennet coagulation studies were conducted in order to understand the renneting capabilities and cheese forming abilities by many researchers. The utilization of ultra-HPH of pressure up to 179 MPa for six passes induced a reduction in the rennet coagulation time as compared to unheated skim milk (Sandra and
Dalgleish, 2007) for different concentration of rennet utilized. They associated this reduction in the rennet coagulation time to an increased removal of κ-casein from the surface of the casein micelles that induce premature interaction and gel formation (Sandra and Dalgleish, 2007). However, at HPH above 200 MPa, an increase in rennet coagulation time (RCT) was observed (Zamora et al., 2007). This increase in RCT has been associated to spreading of free κ-casein making more available for rennet activity that is not associated with the casein micelles, denaturation of β-Lg, change in the initial milk pH, and the changes in concentration of soluble calcium.

The characteristics of cheese produced from UHPH milks with varying fat contents have also been studied. Many of these studies reported an increase in moisture content and cheese yield (Kheadr et al., 2002, Zamora et al., 2007, Zamora et al., 2012b). A corresponding decrease has been observed in the protein content of whey (Zamora et al., 2007). A number of factors have been associated with the increase in bound water in the protein matrix in cheese, which partly contributes to increase in yield. The ultra-HPH pressure increases casein-casein interaction by hydrophobic forces forming a tight protein matrix that can form multiple hydrogen bonds to bind water (Zamora et al., 2011). Whey protein denaturation is also induced during HPH, which incorporates them in the casein matrix by formation of disulphide bonds and hydrophobic interactions (Zamora et al., 2007, Zamora et al., 2011). The denaturation of whey proteins and/or the dissociation of its quaternary structure (multimeric β-Lg) are known to enhance binding of water as well (Kneifel and Seiler, 1993). Another factor that contributes to the increase in yield is the amount of fat incorporated in the cheese. A reduction in the fat globule size is induced with ultra-HPH as compared to raw milk. These fat globules are effectively integrated in to the cheese.
matrix (Zamora et al., 2007). The only exception to increase in yield generally observed with ultra-HPH was the study of Escobar and coworkers indicating a decrease in yield when pressure was increased from 200 to 300 MPa. This was explained to be owing to low whey denaturation and loss of more proteins in whey (Escobar et al., 2011). However, the extent of whey protein denaturation during HPH is still contradictory in different studies. The increase in fat content of the milk utilized for cheese making after ultra-HPH at 300 MPa from 1.8 to 3.6 % fat content also increased the yield by 9 % (Zamora et al., 2012b). Most of the other studies were conducted with milk standardized to 3.5% fat content.

The increase in moisture content and fat content should essentially produce a softer texture of cheese (Lucey et al., 2003). However, it was observed that the type of binding of the water and not the amount of water is responsible for firmness as in ultra-HPH milk cheese the water is in the bound form. Apart from this, the cheese curd firmness is dependent on the inter connectivity of caseins, and the number and strength of bonds linking them (Lucey et al., 2003). The denatured whey proteins can cross-link with caseins, increase the volume of network and reduce the relative movement of strands in network contributing to its firmness (Green et al., 1983, Lucey et al., 2001). Apart from this, the disrupted fat globules can also contribute to firmness of the cheese matrix. The fat globule size reduction on ultra-HPH leads to increased exposure of uncovered fat globule surface to which caseins as well as denatured whey proteins can adsorb (Everett and Olson, 2003, Kheadr et al., 2002, Zamora et al., 2007, Zamora et al., 2011). The proteins on the globule structure interact and are incorporated in the casein matrix (Cano-Ruiz and Richter, 1997). Thus these embedded fat globules lead to formation of thicker strands and coarser matrix forming weak gels (Walstra et al., 1985, Zamora et al., 2007). The rennet-induced
Gels were found to have a brittle texture for ultra-HPH skim milk, as well. An increase in the fat content of the milk used for cheese making (1.8 to 3.6% fat) impaired the hydrogen bonding between caseins and favored hydrophobic interactions of whey proteins and caseins (Zamora et al., 2012b).

**Effect of ultra-HPH on acid gelation and yogurt characteristics.** The present-day trend for the consumption of low fat foods calls for the development of low fat dairy products including yogurt. The possibility of utilizing HPH in the manufacture of yogurt is to possibly eliminate the use of fat replacers including modified starches or proteins that enable the production of low calorie creamy yogurts (Tamime and Robinson, 1999). This idea is based on the ability of HPH to break down food emulsions into smaller particles with narrower particle size distribution (Floury et al., 2004, Hayes and Kelly, 2003). Some studies have been conducted on the effect of ultra-HPH on acid gels and yogurt gels.

Acid gels have been produced from ultra-HPH milks mainly using slow acidifying agents glucono-δ-lactone (GDL) and used as model systems mimicking yogurt gels (Lee and Lucey, 2004, Lucey et al., 1998, Walsh-O’Grady et al., 2001). These acid gels are much easier to control compared to yogurt gels containing live microorganisms. Hernandez and Harte (2008) compared the characteristics of gels from thermally heated milk (TT; 90°C for 5 min), HPH (0 to 350 MPa) and combinations of both (Hernández and Harte, 2008). The gelation time of TT (28 min) was lower than HPH (48 min). This was attributed to the active participation of thermally denatured whey proteins and whey protein aggregated in gel formation (Lucey et al., 1998). The lower gel firmness (G’, storage modulus) of HPH gels (18 Pa) compared to TT gels (260 Pa) was attributed to the extensive rearrangement and aggregation of proteins during HPH (Serra et al.,
2007). However, the whey holding capacity of 350 MPa HPH gel (90 %) was much higher than TT gel (50 %) after 5 min of centrifugation. The increase in retention of water has been associated to the amount of whey protein denaturation and large surface area of disrupted caseins (Serra et al., 2008). A number of yogurt gel studies have been conducted as well. In one such study, the onset of gelation was calculated as the maximum of the first derivative of the NIR signal attenuation obtained from an Optigraphic system. No differences were observed in the gelation time for HPH samples homogenized at different pressures. The increase in aggregation rates has been associated with the increase in casein micelles size after the second stage of homogenization (Serra et al., 2007). They also associated the increase in the rate of aggregation to the decrease in fat globule size, which in turn increases the area of interaction with the milk proteins during gel formation. Another key factor that affects an increase in aggregation rate was explained as increased denaturation of β-Lg (Datta et al., 2005, Desrumaux and Marcand, 2002, Hayes et al., 2005). The denatured whey proteins have also been reported to cluster around fat globules, associate with the casein network and crosslink with it to increase the gel firmness (Serra et al., 2009, Serra et al., 2007). Although many of the HPH studies have explained many of the changes observed in the characteristics of milk especially acid gelation to whey protein denaturation, whey protein denaturation has not been observed by all HPH studies and is subjective to the conditions in each study. Hence, whey protein denaturation has to be ascertained in each study to be able to associate it with the variations in properties observed. In the case of, the heat denatured whey proteins in conventional yogurts are known to produce branched structures. This
provides a fine and homogenous texture to stirred yogurts produced by heat treatment (Serra et al., 2009).

Overall, an understanding of the physico-chemical, functional and technological characteristics of casein micelles and milk proteins are beneficial in enabling their utilization as ingredients in various applications in the dairy industry.

**Summary and objectives**

Over the years the structure of the casein micelles have been extensively researched and modeled, however few aspects of their structure are precisely understood. In order to better understand the properties of the casein micelles we need a better understanding of the structure of the casein micelles. As a step towards understanding the casein micelles, our first objective was to elucidate the size distribution of casein micelles in raw skim milk. Casein micelles have been extensively studied for their binding properties with a number of probes. Most of these studies are conducted using re-assembled casein micelles from sodium caseinates, phosphocaseins and individual caseins, as these are simpler systems to study. We decided to investigate the ability of the casein micelles present in pasteurized skim milk to bind to a hydrophobic probe, vitamin A palmitate. The effect of different processing techniques including thermal and pressure treatments on milk have also been investigated thoroughly. High pressure homogenization is a relatively new technology that was studied till date for homogenization of milk to a pressure of 400 MPa. In the present study we adapted a waterjet to homogenized milk to 500 MPa, which is a very novel approach. We investigated the effect of HPH on the various structural, physicochemical and technological properties of pasteurized skim milk.
References


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CHAPTER 2
SIZE DISTRIBUTION OF CASEIN MICELLES IN RAW SKIM MILK
FROM INDIVIDUAL COWS AS STUDIED USING CRYO-TEM
Abstract
Casein micelles have a complex structure comprising of four caseins, $\alpha_s1$, $\alpha_s2$, $\beta$, and $\kappa$ caseins in the ratio 4:1:3.5:1.5, assembling more than 20,000 casein proteins. Polydispersity is the degree of disparity or variation in size of particles. Casein micelles are known to be highly polydisperse with relative polydispersity ranging from 0.27 to 0.5 as reported by different studies. The size distribution of casein micelles has been studied mostly by dynamic light scattering techniques, which have their own advantages and disadvantages. Cryogenic transmission electron microscopy (cryo-TEM) is a relatively new technique that enables imaging of casein micelles with relatively more clarity and introduction of fewer artifacts as compared to earlier electron microscopy techniques. In the present study we intend to elucidate the size distribution of casein micelles in milk from four cows by cryo-TEM. The raw milk was collected from four cows of first parity, mid lactation and low somatic cell count, and skimmed by centrifugation ($6414 \times g$ for 20 min). The skim milk after dilution (10 µL sample in 500 µL protein free serum obtained by ultrafiltration of skim milk using a 3kDa filter) was dispensed on a grid, frozen in liquid ethane (-182°C), and subjected to cryo-TEM at 200kV. The diameter of casein micelles in the micrographs were measured using ImageJ image analysis software. The relative polydispersity was calculated from the relative standard deviation of the size distribution of casein micelles assuming it to be a Gaussian distribution. The size distribution of casein micelles (x axis - number of micelles as a fraction of the total micelles measured, y axis - diameter of casein micelles) was found to span between 10 to 341 nm (1545 micelles), 24 to 693 nm (1073
micelles), 11 to 514 nm (2621 micelles) and 11 to 532 nm (1891 micelles) for milk from the four cows, respectively. The distribution curve was positively skewed with a tapering tail for large casein micelles of size greater than 350 nm diameter. The casein micelles were observed to have irregular periphery and the varying contrast indicated varied atomic densities within micelles and between micelles. The maximum number of casein micelles existed in the size range of 90 to 110 nm for milk from all the four cows. The relative polydispersity ranged between 0.39 and 0.47 for casein micelles with mean diameters ($D_{10}$) between 112 to 134 nm for the milk from the four cows. The results indicate high level of polydispersity in the casein micelles in milk of every cow. Hence it was possible to effectively utilize cryo-TEM to elucidate the size distribution of casein micelles and visually reconfirm the polydispersity of casein micelles in bovine milk from individual cows.

**Introduction**

Casein micelles are complex quaternary structures formed by the assembly of $\alpha_s1$, $\alpha_s2$, $\beta$, and $\kappa$ casein proteins. They vary in size from 25 to 680 nm based on the range of sizes reported in many studies (Donnelly et al., 1984, Holt et al., 2003, Horne, 1984, Horne and Dalgleish, 1985) and contain more than 20,000 individual $\alpha_s1$, $\alpha_s2$, $\beta$, and $\kappa$ caseins proteins with molecular masses ranging from 19 to 23 kDa in the molar ratio 4:1:3.5:1.5 (Swaisgood, 2003), and micelle mass ranging from $10^6$ to $10^7$ Da (Dalgleish, 2011, Holt et al., 2003). The size of the casein micelles is correlated to the content of different casein fractions and the calcium-phosphate content. For instance, $\kappa$ casein content was shown to decrease with increase in micelles size (Dalgleish et al., 1989, Donnelly et al., 1984, Marchin et al., 2007).
Many factors have been correlated with variation in size of casein micelles. The fact remains that the variability of the casein micelle size distribution is essentially induced in the mammary gland where it is assembled (Farrell Jr et al., 2006). The amino acids are transported to the secretory epithelial cells in the alveoli of the mammary gland and assembled into proteins on the ribosomes of the rough endoplasmic reticulum (Farrell Jr, 1973). The proteins are then transported through the Golgi apparatus where they are eventually phosphorylated, glycosylated, and bound with calcium, in order to form the casein micelles (Farrell Jr, 1973, Keller et al., 1979). The intramolecular association of different caseins, intermolecular associations, association of caseins with calcium and phosphorylation pose multiple equilibria in the system for casein micelle assembly. Apart from the assembling mechanism in the epithelial cells, some level of rearrangement occurs after release of casein micelles from the Golgi apparatus into the alveoli (Brooker and Holt, 1979). The influx of oxygen in milk during milking has also been shown to catalyze the formation of disulphide bonds in κ-casein micelles (Farrell et al., 2003). This indicates that further rearrangement of casein micelles occurs even after assembly due to changes in the milk environment. The complexity of this biological mechanism of casein micelle assembly and transport is the reason for the lack of complete understanding of the formation and structure of casein micelles in milk. This complexity also induces polydispersity of casein micelles in milk, defined as the degree of variability of the size of the casein particles in solution. It is represented using different terms like relative polydispersity, polydispersity and polydispersity index in different studies. The polydispersity of casein micelles in milk have been previously described as being larger compared to other biological systems and smaller than emulsions (Gebhardt et al., 2005, Müller-Buschbaum et al., 2007). Casein micelles have long
been considered a poly-disperse system with polydispersity values ranging from 0.27 to 0.5 from quantification using dynamic light scattering (DLS) techniques (de Kruif, 1998, de Kruif and Huppertz, 2012, Hansen et al., 1996a, Müller-Buschbaum et al., 2007).

The size of casein micelles range between 25 to 680 nm diameter, depending on the various techniques used (Beliciu and Moraru, 2009, de Kruif and Huppertz, 2012, Holt et al., 1978, Horne, 1984, Horne and Dalgleish, 1985). Size of casein micelles in bovine milk is one aspect that has been extensively researched to date in spite of the meager understanding about their structure. However, the biggest concern about these studies is the variations observed in the size distribution of casein micelles in different studies. The reasons attributed to these variations are the effect of the differences in the techniques used and sample preparation methodologies.

The most common technique used for studying casein micelles size distribution is dynamic light scattering (DLS also known as photon correlation spectroscopy or Quasi-elastic light scattering). DLS utilizes laser (monochromatic coherent light) to detect the fluctuation in the molecules in a solutions called Brownian motion. It is measured as the decay rate of the light scattered and the magnitude of the scattering vector. The diffusion pattern of the molecules changes constantly over time owing to the Brownian motion and is determined as the diffusion coefficient quantified using the autocorrelation function (Chu, 2008). This is in turn related to the hydrodynamic size of the micelles. However, the correlation function holds true mainly for monodisperse particles. When DLS is utilized for a polydisperse system like milk with very large and small casein micelles, the integral over all the sizes present is calculated for a continuous distribution to obtain the diffusion coefficient from the autocorrelation function. The inversion of this integral cannot be done to solve for the distribution of particle sizes as it is considered “ill conditioned”. It has also been reported to cause many inconsistencies between samples and among different studies owing to variations in the spacing of the correlation angle, the scattering angle, concentration and method of preparation of the sample and the polydispersity in size of the samples (Alexander and Dalgleish, 2006, de Kruif and Huppertz, 2012, Griffin and Anderson, 1983). These reasons have led to the suggestion of utilizing techniques other than DLS for the estimation of size distributions in the case of polydisperse systems with large particles, like in milk (Alexander and Dalgleish, 2006). A recent study using dynamic light scattering used specific scattering angles in order to improve the uniformity and self consistency of DLS data (de Kruif and Huppertz, 2012) (de Kruif and Huppertz, 2012, de Kruif et al., 2012). On the basis of these assumptions and observations from particle size analysis, de Kruif and Huppertz concluded
that casein micelle sizes from individual cows are monodisperse. They also indicated relative polydispersity values ranging from 0.27 to 0.41. This is contradictory to previous research findings indicating that casein micelles are polydisperse with a wide size distribution range for milks from different cows. Regardless, the current interest in research on casein micelle size is an indicator of the need to better understand the casein micelles in order to utilize their characteristics in milk and milk products.

In the present study, we utilize transmission electron microscopy (TEM) as a technique to further understand the size distribution of casein micelles and eliminate the contradiction existing about their polydispersity. TEM is generally used as a qualitative study and can visually confirm the size variation of casein micelles in bovine milk. Some common issues associated with transmission electron microscopy in the past was the smaller estimates of average micelle size, owing to the shrinkage of micelles during fixation staining and dehydration (Carroll et al., 1968) or the underestimation of the number of large and small micelles (Schmidt et al., 1974). Apart from this, some electron microscopy studies excluded the particles below 25 nm in size, considering them as non micellar caseins (Holt et al., 1978, McGann et al., 1980). Cryogenic transmission electron microscopy (Cryo-TEM) is a relatively new EM technique. In this technique a thin layer of the sample suspension is quickly frozen in a cryogenic fluid, retaining the amorphous nature of the aqueous samples within the thin layer of vitreous ice formed. It does not require staining or dehydration for visualization, instead utilizes the contrast produced by the coherent interference of the scattered and transmitted electron beams (Karlsson et al., 2007, Knudsen and Skibsted, 2010, Marchin et al., 2007, Trejo et al., 2011). Thus, it induces the least amount of artifacts in the samples compared to other electron microscopy techniques utilized
previously (McMahon and McManus, 1998). Owing to the less invasive nature, cryogenic TEM (cryo-TEM) can be utilized for quantifying sizes and imaging the internal structures of complex structures, like casein micelles. The development and utilization of other new techniques, like diffusing wave spectroscopy, could also be beneficial in complementing cryo-TEM results. Hence, the objective of our study was to investigate the polydispersity of casein micelles using cryo-TEM, which can provide a visual observation of the size distribution of casein micelles in bovine milk from individual cows.

**Materials and methods**

*Sample collection and preparation*

Raw milk samples were collected from the Little River Animal Facility at the University of Tennessee, Knoxville. A group of four cows were chosen of first parity, mid-lactation and low somatic cell count. Details of the days of lactation, milk yield, milk fat, milk protein and somatic cell count are given in Table 2.1. The samples were collected during the morning milking. Fat was separated by centrifugation (6,414 x g for 20 min at 15 °C) and the fat layer on top was removed manually using a spatula. The skim milk was refrigerated at 4°C until microscopy preparation.

*Cryo-TEM and Image analysis*

Cryo-TEM was performed on the raw skim milk samples after diluting with protein free serum (PFS). The PFS was obtained by tangential ultrafiltration (PLBC Prep scale TFF Cartridge with a molecular weight cutoff of 3 kDa, Millipore, Billerica, MA) of pasteurized skim milk, preserved with 0.07% sodium azide and stored at -4°C. The PFS was thawed, centrifuged at 5,200 × g for 20 min and filtered using a 0.45 μm PVDF filter prior to sample preparation. The milk dilutions
were prepared in 1mL microcentrifuge tubes with 10 µL of milk in 500 µL of PFS. The diluted milk sample (5 µL) was then dispensed on to a 200 mesh holey grid (Lacey carbon film grid, Structure Probe, Inc., Westchester, PA) held in place for cryo-sample preparation on Gatan Cp3 Cryoplunge™ (Gatan Inc., Pleasanton, CA). The samples were blotted for 3.5 sec. The grids with samples were frozen by plunging in to liquid ethane at -182°C and then transferred to a Gatan cryo-sample holder. The frozen samples were then observed under Zeiss Libra 200 MC electron microscope at 200 kV (Carl Zeiss Inc., Fort Lauderdale, Florida) with a 2K/2K camera (Gatan Ultrascan US1000XP, Gatan Inc., Pleasanton, CA) and imaging unit using the Digital Micrograph™ software (Gatan Inc., Pleasanton, CA).

The density of the casein micelles were visually compared by assuming direct relation of contrast between different micelles and within micelles with atomic density (Dubochet et al., 1983). The diameter of the casein micelles were measured using ImageJ image analysis software package (National Institutes of Health, Bethesda, Maryland, http://imagej.nih.gov/ij/). The micelles in every image were labeled before measuring the diameter based on the following criteria:

- Micelles should be easily distinguishable (not less than 10 nm diameter) and dispersed with definite surface boundary, should not have electron beam damage (usually identified as white spots or smudges), should be within the holes of the grid and in between the holes of the carbon network and not on top of it.

- Micelles should not overlap each other.

After labeling the micelles, the diameter of each micelle was measures based on the following criteria:
The line connecting the outermost points on the opposite sides through the middle point of spheric micelles was measured as the diameter of the micelles.

In the case of ovoid micelles, the diagonal of the ovoid was measured as the diameter of the micelles.

In the case of an irregular micelle, diameter of the biggest circular area with maximum filled space was considered for measurement of diameter.

Data analysis
The numbers of micelles measured for diameter from the milk from the four cows were 1545, 1073, 2621 and 1891, respectively. The frequency of micelles of a particular radius was expressed as a percentage and represented as the size distribution curve with percentage (y-axis) against radius (x-axis) of micelles. The arithmetic or number mean ($D_{10}$) and standard deviation of the distribution curve were utilized to calculate the relative polydispersity of the casein micelles sizes in the milks from the individual cows. The size distribution of the casein micelles has been referred to be a log normal distribution by previous studies represented by the following equation (de Kruif, 1998):

$$f_{ln}(d) = \frac{1}{(d\beta \sqrt{2\pi})} \times e^{-\frac{\ln(d) - \ln(D_{10})}{\beta \sqrt{2}}}$$

where,

$d$ – diameter in nm

$D_{10}$ – Arithmetic or number mean of particle diameter expressed as, $D_{10} = \frac{\sum_{i=1}^{n} di}{n}$

$n$ – number of micelles measured for diameter
β – measure of relative polydispersity which is related to standard deviation (σ) for a Gaussian or normal distribution as given by:

\[ \beta \approx \sqrt{\ln \left( \frac{\alpha}{D_{10}} \right)^2 + 1} \]

The D_{10} and the β values were utilized to construct the fitted log-normal distribution curve for the size distribution of casein micelles from the milk of each cow, using the function for log-normal curve given above.

**Results and discussion**

*Cryo-TEM of casein micelles*

The casein micelles exhibited spherical and ovoid shapes with irregular surfaces. Many of the micelles were irregular, which is consistent with the observations by other research groups (Marchin et al., 2007, Trejo et al., 2011). The structure of the casein micelles is an entangled network and has an irregular periphery. Scanning electron microscopy (Dalgleish et al., 2004, McMahon and McManus, 1998) and TEM (Marchin et al., 2007, McMahon and McManus, 1998, Schrader et al., 1997) techniques have confirmed the irregular surface of the casein micelles. This irregularity of the surface has been mainly attributed to the outward hairy layer of κ-caseins. The conformational mobility of the C terminal end of κ-caseins has been shown by 3¹P-NMR (Thomsen et al., 1995), indicating their position towards the surface. The latest study using cryo-TEM tomography produced a model of casein micelles with an irregular surface and an entangled inner structure (Trejo et al., 2011).

*Figures 2.1 and 2.2* shows the presence of low and high density areas within the casein micelles. The low density areas indicate the presence of cavities and channels. Presence of water filled
channels of approx. 5nm size and cavities of 20 to 30 nm diameter have been previously reported (McMahon and Oommen, 2008, Trejo et al., 2011). The gaps in the protein structure of the micelles had been postulated earlier (Dalgleish, 2011). The high density areas observed in Figures 2.1 and 2.2 can be associated with the interactions between caseins and colloidal calcium phosphate (CCP) complexes, which are referred to as ‘nanoclusters’ within the casein micelles. The dark spots visible in Figure 2.2 indicate the colloidal calcium phosphate nanoclusters. The sizes of CCP nanoclusters range between 6 and 12 nm, as reported earlier (Trejo et al., 2011). A few studies have utilized 3D electron maps or image analysis using density filters obtained from cryo-tomography to interpret the density of proteins (Sharp et al., 2012, Trejo et al., 2011). There are TEM studies that utilize the contrast in the images to interpret and distinguish structural variations and bilayers (Berlepsch et al., 2014, Kellermann et al., 2004). Contrast has been described as being directly proportional to density of atoms in TEM images (Dubochet et al., 1983, Zhu et al., 2010).

Figures 2.2 and 2.3 portray the variety of sizes of casein micelles in bovine milk (56 nm to 522 nm in Figure 2.2 and from 18 nm to 273 nm in Figure 2.3). These images are a representation of the different sizes in which casein micelles exist in milk. These images also indicate the presence of different densities for casein micelles of similar sizes. This is observed for micelle labeled “3” and “6”, having diameters of 188 nm and 183 nm, and micelle numbers 1, 12 and 16, having diameters of 161 nm, 156 nm, and 169 nm, respectively (Figure 2.2). The density of casein micelles in co-mingled milks have been reported to be different (Holt, 1975, Holt et al., 1978). This variation could be associated with natural variations in the conditions of bioassembly in the bovine physiological system of different cows. In our study however, the different densities
existed among casein micelles of the same size within the milk samples from an individual cow. This has been demonstrated earlier by the overlap of particle size ranges for different pellets or sections separated on the basis of weight and molecular weight by using techniques like differential centrifugation (Horne and Dalgleish, 1985) and sedimentation studies (Dewan et al., 1974). Apart from this, rearrangements occur after expulsion from the alveoli in the lumen and mammary cistern, during and after milking (Farrell Jr et al., 2006, Jenness, 1974). Another factor that could induce changes in the casein composition of micelles is the storage conditions, especially the temperature of storage and time of exposure to a particular temperature (Beliciu and Moraru, 2009, Davies and Law, 1983). These studies indicate that at low temperature storage below 10°C, a leaching out of caseins from the micelle reduces the hydrophobic interactions within and thus increases their size. At temperatures as high as 50°C there is a reversal of this phenomenon reincorporating the caseins in to the micelle, packing them more tightly together and reducing its micelle size. In the present study, raw skim milk samples were kept below 10°C until microscopy. Overall variation in density of casein micelles of similar size from the same cow could also be a normal scenario even under controlled conditions.

Size distribution of casein micelles
The size distribution of casein micelles from raw skim milk from four cows based on their diameter is given in Figure 2.4, 2.5, 2.6 and 2.7. The casein micelles size distribution of the four cows indicated a similar pattern. These data are not sufficient for any conclusive evidence on the similarity of size distribution of casein micelles in different cattle types, parity, time of milking and duration in lactation. However, the four cows used for the study exhibited similar size distribution patterns for the casein micelles present in their milk. de Kruif and coworkers have
reported that the size of casein micelles in milk reconstituted from low heat skim milk powder studied by DLS backscattering technique showed variations between cows and batches (De Kruif et al., 1989). The similarity of size (R_h) from DLS between milkings and lactations over a period of three years in an individual cow has been reported by de Kruif and Huppertz (2012). The maximum number of casein micelles ranged in the diameters of 90 to 110 nm for the milk from the four cows (Figure 2.4, 2.5, 2.6 and 2.7). Holt and coworkers (1978) also observed 80 to 100 nm diameter of casein micelles when they used freeze etching for analyzing the size distribution of casein micelles in milk from two cows by TEM. Other studies have shown different size distribution curves using different electron microscopy techniques (Carroll et al., 1968, Nitschmann, 1949, Rose and Colvin, 1966, Schmidt et al., 1974). Many of these studies show underestimation of micelles with diameters less than 160 nm. Cryo-TEM technique induces the least amount of disruption to the micelle structure. In order to keep the micelles in their native state, PFS obtained from the ultrafiltration of milk was utilized for diluting the milk samples. The use of casein depleted ultrafiltrate was stated to be the best solvent for size analysis studied by Beliciu and Moraru (2009). The studies conducted using DLS also indicates that the maximum number of casein micelles to exist in the range of 90 to 110 nm diameter (Horne and Dalgleish, 1985). Thus in terms of the maximum number fraction of casein micelles, the range of 90 to 110 nm obtained in the present study is in consensus with results obtained from other studies.

The size distribution of casein micelles ranged from 10 to 341 nm, 24 to 693 nm, 11 to 514 nm and 11 to 532 nm for the milk from the four cows (Table 2.2). These size ranges are very similar to the distributions reported in many light scattering studies. The size range was between 25 and
680 nm considering most of the published information about casein micelles to date (Dewan et al., 1973, Donnelly et al., 1984, Fox and Brodkorb, 2008, Holt, 1975, Holt et al., 1978, Horne and Dalgleish, 1985, Lin et al., 1971). Hence, the casein micelles size distribution obtained in this study is comparable to other techniques including DLS and electron microscopy. The size distribution curve was positively skewed and observed to have a tapering tail region with low number fractions for micelles with diameter above 350nm (Figure 2.4, 2.5, 2.6 and 2.7). Such a skewed distribution has been repeatedly reported for TEM studies of casein micelles with large population of smaller micelles (Dewan et al., 1974, Holt et al., 1978, Lin et al., 1971, Schmidt et al., 1974). Similar tail regions have also been reported by Holt et al. (1978) in their study of casein micelle size distribution using electron microscopy by freeze etching. Their estimation of these particles was 10% of the weight distribution curve indicating that these micelles tend to influence the weight distribution size averages produced by DLS. This underestimation of larger micelles were correlated to the lower mean sizes obtained for casein micelles as obtained by TEM compared to DLS techniques. According to Holt and group (1978), the reason is the inability to detect or characterize very large micelles by TEM. Another factor that could be responsible for the consistent higher mean micelles sizes using DLS technique could be because light scattering is proportional to the square of the molecular weight of the scattering particle. Thus, the intensity of light scattering is higher for large micelles and it affects the correlation function and the diffusion coefficient. This in turn could bias the $R_h$ calculated from the diffusion coefficient to the larger side in DLS studies (Griffin and Anderson, 1983). Moreover, the number distribution used in TEM favors smaller micelles (Lin et al., 1971). The conversion of micelle size distribution to comparable forms indicates similarity in the average
micelle size obtained from EM and DLS (Holt et al., 1978, Lin et al., 1971). The presence of such large micelles as indicated in the tail region of the size distribution has been compared to the size of the Golgi vesicles, where these micelles are assembled (Farrell Jr et al., 1990, Farrell Jr et al., 2006). Some of the casein micelles in the tail region of the distribution were found to be much bigger than these Golgi vesicles. This has been associated to rearrangement in the duct and during and after milking (Farrell Jr et al., 2006, Holt et al., 1978). Despite of the cumbersome nature of this technique, cryo-TEM was found effective for quantifying the size distribution of casein micelles.

**Polydispersity**
The relative polydispersity indicates the width of the particle size distribution. The relative polydispersity values for the casein micelles from the four cows varied from 0.39 to 0.47 with mean diameter ($D_{10}$) ranging between 112 to 134 nm (Table 2.2). This provides evidence of the high polydispersity that exists within the casein micelles in bovine milk. The existence of this polydispersity has been correlated to the complex multiequilibrated assembly system for casein micelles in the mammary gland. A small angle neutron scattering study indicated a polydispersity index of 50% (equivalent to relative polydispersity of 0.5) for the radius of casein micelles with an average radius of 100 nm (Hansen et al., 1996a). Recently a study conducted by de Kruiif and Huppertz (2012) have described casein micelles to be very monodisperse for individual cows despite of reporting a relative polydispersity of 0.27 to 0.41 in their study. The polydispersity of protein solutions less than 0.2 is considered a monodisperse solution. The authors could be referring to the lack of variation between milkings and over time for the same
cow. Hence, the polydispersity of the casein micelles remains uncontested and is observed in the size distribution of casein micelles in milk from individual cows.

**Conclusions**

Casein micelles have a tangled porous structure with an irregular surface. The micelle internal structure consists of proteins, colloidal calcium phosphate nanoclusters, channels and crevices. These were visible as light and dense areas in the cryo-TEM images. The casein micelle sizes in milk from individual cows indicate a broad distribution with high polydispersity between 0.39 to 0.47. The mean diameter for each cow falls in the range of 112 to 134 nm. Previously EM techniques have been utilized to determine the size distribution of casein micelles in milk, cryo-TEM has been used for determining the size distribution for the first time. The results from the study indicate that it can be effectively utilized to elucidate the size distribution of casein micelles. Moreover, the results reconfirmed the presence of high polydispersity in the sizes of casein micelles even in the milk from individual cows.

**Acknowledgements**

We would like to acknowledge the Little River Animal facility associated with the University of Tennessee for providing the raw milk required for the study. We would also like to acknowledge Dr. John Dunlap at the Advanced Microscopy and Imaging Center, Science and Engineering Research Facility, The University of Tennessee for providing guidance and expert advice for cryo-TEM technique.
References


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Table 2.1: Details of milk yield, fat percentage, protein percentage and somatic cell count for milk from the four Holstein Friesian cows used in the present study

<table>
<thead>
<tr>
<th>Cow No.</th>
<th>Age on date of milking (days)</th>
<th>Days in milking</th>
<th>Milk yield</th>
<th>Fat%</th>
<th>Protein%</th>
<th>Somatic cell count (x1000 cells/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1075</td>
<td>147</td>
<td>78.9</td>
<td>2.9</td>
<td>2.7</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>952</td>
<td>149</td>
<td>66.9</td>
<td>3.8</td>
<td>3.2</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>897</td>
<td>167</td>
<td>77.1</td>
<td>4.4</td>
<td>3.1</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>944</td>
<td>126</td>
<td>77.9</td>
<td>4.1</td>
<td>2.9</td>
<td>13</td>
</tr>
</tbody>
</table>
Figure 2.1: Cryo-TEM images of casein micelles at different magnifications. The black arrows indicate dark particles that are the colloidal calcium phosphate nanoclusters.
Figure 2.2: Cryo-TEM image showing casein micelles in milk with number labeling for each micelle and the corresponding diameters indicated in the table
Figure 2.3: Cryo-TEM image displaying the wide variety of sizes of casein micelles
Figure 2.4: Casein micelle size distribution as percentage of micelles against radius of micelles for milk from cow 1. Blue dots indicate the size distribution and the red dots with line indicate the fitted log normal curve. The inset shows the arithmetic mean of radius ($R_{10}$), standard deviation ($\sigma$) and correlation coefficient of the size distribution and fitted log-normal curve.
Figure 2.5: Casein micelle size distribution as percentage of micelles against radius of micelles for milk from cow 2. Blue dots indicate the size distribution and the red dots with line indicate the fitted log normal curve. The inset shows the arithmetic mean of radius ($R_{10}$), standard deviation ($\sigma$) and correlation coefficient of the size distribution and fitted log-normal curve.

- $R_{10} = 67$ nm
- $\sigma = 32$ nm
- Corr. = 0.99
Figure 2.6: Casein micelle size distribution as percentage of micelles against radius of micelles for milk from cow 3. Blue dots indicate the size distribution and the red dots with line indicate the fitted log normal curve. The inset shows the arithmetic mean of radius ($R_{10}$), standard deviation ($\sigma$) and correlation coefficient of the size distribution and fitted log-normal curve.
Figure 2.7: Casein micelle size distribution as percentage of micelles against radius of micelles for milk from cow 4. Blue dots indicate the size distribution and the red dots with line indicate the fitted log normal curve. The inset shows the arithmetic mean of radius (R\textsubscript{10}), standard deviation (\sigma) and correlation coefficient of the size distribution and fitted log-normal curve.

R\textsubscript{10} – 64 nm
\sigma – 32 nm
Corr. – 1.0
Table 2.2: The average (arithmetic mean) casein micelles diameter ($D_{10}$), standard deviation ($\sigma$), relative polydispersity ($\beta$) and the minimum and maximum diameter of the casein micelles in the milk from the four cows

<table>
<thead>
<tr>
<th>Cows</th>
<th>$D_{10}$ (nm)</th>
<th>$\sigma$ (nm)</th>
<th>$\beta$</th>
<th>Smallest (nm)</th>
<th>Largest (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow 1</td>
<td>125</td>
<td>51</td>
<td>0.389</td>
<td>10</td>
<td>341</td>
</tr>
<tr>
<td>Cow 2</td>
<td>134</td>
<td>63</td>
<td>0.448</td>
<td>24</td>
<td>693</td>
</tr>
<tr>
<td>Cow 3</td>
<td>112</td>
<td>53</td>
<td>0.450</td>
<td>11</td>
<td>514</td>
</tr>
<tr>
<td>Cow 4</td>
<td>128</td>
<td>63</td>
<td>0.466</td>
<td>11</td>
<td>532</td>
</tr>
</tbody>
</table>
CHAPTER 3A
BINDING OF VITAMIN A BY CASEIN MICELLES IN COMMERCIAL SKIM MILK
A version of this chapter was originally published by M. S. Mohan, J. L. Jurat-Fuentes, and F. Harte:

This chapter is a lightly revised version of the above paper submitted to the Journal of Dairy Science. The use of “our” in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the sample preparation, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the manuscript writing.

Abstract
Recent studies have shown that re-assembled micelles formed by caseinates and purified casein fractions (αs and β) bind to hydrophobic compounds, including curcumin, docosahexanoic acid (DHA), and vitamin D. However, there has been limited research on the binding of hydrophobic compounds by un-modified casein micelles in skim milk. In the present study, we investigated the ability of casein micelles in commercial skim milk to associate with vitamin A (retinyl palmitate), a fat soluble vitamin commonly used to fortify milk. Milk protein fractions from different commercially available skim milk samples subjected to different processing treatments including pasteurized, ultrapasteurized, organic pasteurized and organic ultrapasteurized milks were separated by Fast Protein Liquid Chromatography (FPLC). The fractions within each detected chromatograph peak were combined and freeze dried. Silver staining of SDS-PAGE gels was used to identify the proteins present in each of the peaks. The skim milk samples and peaks were extracted for retinyl palmitate and quantified against a standard using normal phase high performance liquid chromatography (NP-HPLC). Retinyl palmitate was found to associate with the section of skim milk containing caseins, while the other proteins (BSA, β-Lg, α-La) did not show any binding. The different milk samples contained 2.48, 1.59, 2.23 and 1.82 µg of retinyl palmitate per mL of milk-1, milk-2, milk-3 and milk-4, respectively. The casein sections
contained between 14 to 40% of total retinyl palmitate in the various milk samples tested. The variation in the retention of vitamin A by caseins was probably explained by differences in the processing of different milk samples, including thermal treatment, the form of vitamin A emulsion used for fortification, and the point of fortification during processing. Our results indicate that unmodified casein micelles have a strong intrinsic affinity towards the binding of vitamin A used to fortify commercially available skim milks.

**Introduction**

Retinyl palmitate (LogP > 4.5) is commonly used for Vitamin A fortification in commercial whole, 2% and skim milk. According to the code of federal regulations (21 CFR 131.110), vitamin A content of fortified milk must be greater than 2000IU/qt (1.2 µg/mL). Vitamin A is normally present in bovine milk at an average amount of 1347 IU/qt (USDA-ARS, 2011). The per capita milk consumption of 83.9 L/person provides very low amount of vitamin A in the diet (IDF, 2007). This makes it essential to fortify milk with vitamin A to help meet the total daily requirement of 700 - 900 µg/day for men and women (Food and Nutrition Board Institute of Medicine, 2001). Vitamin A is added in milk as a mixture of oils and emulsifiers. A typical commercial source for vitamin A and D fortification in the dairy industry contains corn oil, sorbitan monooleate, polysorbate 80, retinyl palmitate (vitamin A), and cholecalciferol (vitamin D₃), with 1 mL containing 200,000 IU vitamin A and 40,000 IU vitamin D₃. Although the addition of oils and emulsifiers makes it possible to for vitamin A to be dispersed in the continuous serum phase of milk, milk proteins are also known to bind hydrophobic compounds. About 80% of the total 3.6% of proteins in milk are caseins. Caseins in milk exist as supramolecular structures called casein micelles. Their sizes range from 50 to 500 nm diameter,
with an average of 120 nm (Fox and Brodkorb, 2008). The structure of the casein micelles has been widely studied and disputed (Dalgleish, 2011). There is a general consensus that the structure of the casein micelles is mainly stabilized by hydrophobic and ionic interactions (Holt et al., 2003, Horne, 1998). The latest studies confirm the presence of a number of cavities (~ 20 to 30 nm diameter) and channels (~ greater than 5 nm diameter) within the casein micelle structure (Trejo et al., 2011). Re-assembled casein micelles produced by various methods were shown to entrap hydrophobic probes like triclosan, vitamin D\textsubscript{2} and DHA (Roach et al., 2009, Semo et al., 2003, Zimet et al., 2011). Micelles formed from β-caseins act as a drug delivery system, by entrapping a hydrophobic anticancer drug, mitoxantrone within it (Shapira et al., 2010). Milk proteins especially caseins were shown to bind to green tea flavanoids by their decrease in hydrophobicity on binding to these hydrophobic polyphenols (Yuksel et al., 2010). Another study evaluated the ability of native caseins to form micelles and complex with curcumin, as a potential drug nanocarrier against cancer cells (Sahu et al., 2008). Reassembled α\textsubscript{s1}- casein micelles also bind with curcumin (Sneharani et al., 2009). All these studies show the ability of reassembled casein micelles or individual caseins to bind hydrophobic molecules. This ability of casein micelles to bind has been associated with the existence of hydrophobic forces, cavities and channels within the micelle structure.

Milk whey proteins also exhibit non-specific affinity to bind hydrophobic molecules. β-lactoglobulin has been shown to bind to vitamin D\textsubscript{3} (Forrest et al., 2005), retinol (Dufour and Haertlé, 1991), DHA (Zimet and Livney, 2009), and polyphenolic compounds, e.g., resveratrol from grapes (Hemar et al., 2011) and epigallocatechin gallate from green tea (Kanakis et al., 2011); α-lactalbumin was shown to complex with retinol and palmitic acid (Puyol, 1991). BSA
can form a water soluble complex with retinol, possibly acting as an in vivo carrier of excess free retinol introduced into the circulation (Futterman and Heller, 1972). BSA is also known to bind to ascorbates, which are precursors of vitamin C (Oelrichs et al., 1984).

In the present study, we investigate the ability of casein micelles and whey proteins, to entrap vitamin A without altering the natural conditions in which they are present in commercially available heat treated skim milk samples. Heating induces conformational changes in proteins which affect their binding to hydrophobic molecules (Kulmyrzaev et al., 2005). Whey proteins denature when milk is processed to temperature above 60°C and form casein-whey proteins complexes (Donato and Guyomarc’h, 2009). The objective of this study was to investigate the ability of the various protein fractions in fortified commercial skim milks to bind retinyl palmitate. The thermal treatments of these skim milks may promote conformational changes that affect the binding properties of proteins. Our aim is to study the ability of fractions of milk proteins in commercially available skim milk samples to bind the vitamin A fortified in them.

**Materials and methods**

*Milk samples and protein free serum*

Four commercially available skim milks fortified with vitamin A (as retinyl palmitate) were studied. Skim milk has less than 0.5% fat present in it (21 CFR 131.110). These skim milk samples were chosen based on the following processing criteria: pasteurized, organic pasteurized, ultra-pasteurized, and organic ultra-pasteurized. Protein free serum was prepared from pasteurized skim milk using 3 kDa molecular weight cut off cellulose tangential flow ultrafiltration system (PLBC Prep scale TFF Cartridge, Millipore, Billerica, MA), preserved with 0.07% (w/v) sodium azide, and stored at 4°C (referred as PFS).
**Size exclusion chromatography**
Skim milk samples (2 mL) were separated into its different protein fractions by using a size exclusion chromatography (SEC) column (HiLoad 16/60 Superdex 200 prep grade, GE Life Sciences, GE Health Care, Piscataway, USA) connected to a FPLC unit (AKTA, GE Life Sciences, Piscataway, USA). Ice-cold PFS was used as the mobile phase. Separation was achieved using a flow rate of 1.0 mL/min and 1 mL fractions were collected using an automatic fraction collector (Frac-950, GE Life Sciences, Piscataway, USA) after 0.3 column volumes for a total elution volume of 1.5 column volumes (~ 180 mL). The peaks detection was done using UV light at 280 nm. Collected fractions within the same peak (protein milk sections) were pooled together and freeze dried.

**HPLC**
Extraction of vitamin A from skim milk samples was done by liquid-liquid extraction as detailed in the AOAC method 2002.06 (Official Methods of Analysis, 2006). Briefly, 2 mL milk samples were transferred into 30 mL centrifuge tubes to which 5 mL of absolute ethanol was added, vortexed for 30 sec and left to stand for 5 min. Then, 5 mL hexane was added and samples subjected to 3 series of vortexing (30 sec) and standing (2 min). In the final step 3 mL DI water was added to samples, vortexed for 5 sec, and centrifuged for 10 min at 633×g (RC-5B centrifuge, SH-3000 rotor, Sorvall, Buckinghamshire, UK). The top hexane layer in the tubes was then transferred to amber HPLC vials and loaded to the HPLC system equipped with a diode array detector (1200 series, Agilent, Santa Clara, CA). HPLC was performed using a normal phase silica 4.6 x 250 mm column (Zorbax Rx-SIL, Agilent, Santa Clara, CA) set at 23°C. The mobile phase was conditioned hexane prepared from 99.85% v/v of wet hexane (500 mL hexane conditioned with 25 mL distilled water overnight) and 0.15% v/v of Isopropanol, and was
pumped at flow rate of 1 mL/min. The automatic injection system injected 100 µL of sample into the system and the vitamin A (retinyl palmitate) peak was detected between 2.5 and 3.0 min of retention by absorbance at 325 nm. The vitamin A content was calculated using the standard curve constructed using retinyl palmitate standard (#R1512, Sigma-Aldrich, St. Louis, MO) and expressed as µg/mL of milk. The freeze dried sections were diluted with 1 mL distilled water and extracted using the method described above.

**SDS-PAGE**
The solid and liquid phase obtained after removing the hexane layer for vitamin A extraction procedure from each section of the milk samples was mixed thoroughly. The SDS-PAGE was performed by a method adapted from Laemmli (1970). From this mixture, 150µL was mixed with loading buffer (0.5 M Tris-HCl pH 6.8, glycerol, 10% (w/v) SDS, β-mercaptoethanol, 0.5% (w/v) bromophenol blue in water; Fisher Scientific, Fair Lawn, NJ). These samples were heated in a water bath at 95°C for 5 min, then loaded on an electrophoresis gel (12%Tris-HCl, 12 wells, Ready Gel, BIO-RAD, Hercules, CA) along with 10 µL pre-stained standard (kaleidoscope, #161-0324, BIO-RAD, Hercules, CA). The gel was subjected to an electric current of – 200V for 38 min and then silver stained. After sufficient stain was observed, staining was stopped and the gel dried (GelAir Drying System, BIO-RAD, Hercules, CA).

**Statistical analysis**
Statistical analysis of data was done using MIXED procedure of SAS (Version 9.3, SAS Institute, 2003) in a completely randomized design. Analysis of variance was used to calculate the mean and standard error for retinyl palmitate concentration in samples of skim milk and skim
milk casein sections. Two replicate samples of commercially available skim milk with the same processing criteria from different brands were chosen for analysis. Duplicate samples from different containers of skim milk from the same manufacturer and batch, having undergone the same processing conditions were studied for each replicate sample of milk.

**Results and discussion**

*Size exclusion chromatography and SDS-PAGE*

The FPLC chromatograms indicated the presence of four different fractions of proteins in the skim milk samples based on their size/ molecular weight (Figure 3A.1 to 3A.4). In order to identify the proteins, the fractions in each peak were pooled together as sections, freeze dried and subjected to SDS-PAGE. The SDS-PAGE results indicate the presence of mainly caseins in the peak 1 (Section 1), BSA in peak 2 (Section 2), β-Lg in peak 3 (section 3), and α-La in peak 4 (section 4), although each peak contained some fractions of other proteins as well (Figure 3A.1 to 3A.4). Similar results were also observed in previous studies conducted in our laboratory (Trejo, 2009). The peak 5 with a shoulder indicated the presence of another proteinaceous fraction; however the SDS-PAGE did not show any proteins in this peak. Other studies conducted by Guyomarc’h and group (2003) have also observed similar non-protein fractions on absorbance detection at 280nm for size exclusion chromatography. FPLC chromatograms of milk-2 sample showed β-Lg and α-La whey proteins eluting in the same peak. This was also accompanied by the absence of peak 4 for this skim milk sample in Table 3A.1. This could be a result of the association of whey proteins to casein micelles due to the specific thermal treatment in milk-2. There are number of studies supporting the unfolding, aggregation and polymerization of β-Lg and α-La whey proteins in milk on being subjected to high temperature treatment.
Apart from the temperature, differences in the heating method and time required to reach the desired temperature, and actual retention time also cause such variations (Oldfield et al., 1998). Section 1 containing mostly caseins has been referred to as casein section of skim milk. The presence of small fractions of whey proteins along with the casein fraction in peak 1 is a result of the heat treatment the skim milk samples had been subjected to, as reported in other studies (Anema and Li, 2003, Singh, 1995). Peak 2 contains fractions of β-casein, κ-casein and whey proteins apart from BSA. The presence of these fractions indicates the formation of soluble aggregates on being exposed to the heat treatments in the skim milk samples (Dalgleish et al., 1997). Table 3A.1 indicates that the Peak 1 in all milks containing mainly caseins ranged from 81 – 89 % of the total peak area for all proteins in the milk. There are no significant differences in the percentage peak area of peak 1 and 2 in all the milk samples. Hence, despite of the slight variations in peak 3, all the milk samples had similar protein fractions in all the peaks as observed by size exclusion chromatography. Although the absorbance peaks in the FPLC chromatograph are dependent on the extinction coefficients of each protein constituting the peak, Table 3A.1 gives an idea about the effect of the different processing conditions on the protein make up of each FPLC peak.

**Vitamin A in skim milk and its sections**

The retinyl palmitate content of skim milk samples were quantified by AOAC 2002.06 methods using HPLC (Table 3A.2). The highest retinyl palmitate content was found in milk-1 (2.48 ± 0.02 µg in 1 mL of milk) and lowest in milk-2 (1.59 ± 0.02 µg in 1 mL of milk). The retinyl palmitate content of milk-3 and milk-4 are 2.23 ± 0.02 µg and 1.82 ± 0.02 µg in 1 mL of milk, respectively. These variations might be due to the type and amount of vitamin A added as well as
the processing method for incorporation by the different companies. Earlier studies have also shown that there are wide variations in the amount of vitamin A fortified in fluid milk available on the market (Murphy et al., 2001).

The different freeze dried sections (sections 1 to 8) obtained from the FPLC of skim milk samples were quantified for the retinyl palmitate content. Vitamin A was detected only in the casein section of the skim milk samples. The eluate obtained before the elution of peaks was absent of any retinyl palmitate content. The caseins in milk have also been reported to be associated with other hydrophobic probes, e.g., vitamin D₂, DHA, curcumin and green tea flavanoids (Sahu et al., 2008, Semo et al., 2003, Yuksel et al., 2010, Zimet et al., 2011). Cavities, channels, and hydrophobic forces within casein micelles have been associated with its ability to bind small hydrophobic probes (Trejo et al., 2011). The retinyl palmitate content of the casein section was 0.915, 0.222, 0.555, and 0.734 (± 0.03) µg/mL of milk-1, milk-2, milk-3, and milk-4, respectively (Table 3A.2). Earlier studies on the structure of casein micelles and its binding properties indicate the strong chances of the retinyl palmitate being trapped within the casein micelles. Some studies have shown that small fractions of fat and phospholipids which can bind hydrophobic compounds, are also found along with the casein micelles after size exclusion chromatography (Corredig and Dalgleish, 1998). In addition to association to this, milk fat globule membranes (which mainly consists of phospholipids) have been shown to associate with whey proteins as well (Corredig and Dalgleish, 1996). Another possibility is the formation of small emulsion droplets of retinyl palmitate which may elute along with casein micelles in the first peak. In all such cases the chances are that different size emulsion droplets, phospholipids or fat droplets could be eluted out along with the other proteins in the other peaks of the FPLC.
chromatograph as well. In order to confirm the association of retinyl palmitate to casein micelles, further studies need to be conducted.

Although the casein section retained an average of 29% and a maximum of 40% of the total retinyl palmitate in the skim milk, the percent of total vitamin A retained in the casein was variable among the various commercial milks used (Table 3A.2). The highest amount of retinyl palmitate was retained by the casein sections of the milk-4 (40.4 ± 2.13 %) and milk-1 (37.0 ± 2.13 %), which were not significantly different. The lowest amount of retinyl palmitate was retained by the casein section of the milk-2 (14.0 ± 2.13 %). Milk-3 retained about 24.9± 2.13 % of retinyl palmitate content in the casein section as compared to the total amount in the skim milk. These results account for only part of the retinyl palmitate as being retained in the casein section. In a study conducted by Semo et al. (2003), the total retention of vitamin D₂ in casein and serum phase was up to 85% of the amount originally added. The reason for this incomplete retention is not yet known.

The variations in the retention of retinyl palmitate in the casein section of different skim milks could be associated with a number of reasons. The type of vitamin A emulsion added to the milk is one among them. Studies have also shown that combination of different process parameters like temperature, pH and pressure on skim milk, affects the binding properties of casein micelles (Orlien et al., 2010). Heating of milk alters the ability of casein micelles to bind to curcumin (Yazdi and Corredig, 2011). In the present study however, the effect of type of processing of the milks, including thermal processing and homogenization, on the retinyl palmitate content is not known. Moreover, earlier surveys have shown that variations exist in the processing points at which retinyl palmitate is added during the processing of milk (Hicks et al., 1996).
Conclusion
Casein micelles in fortified commercial skim milk were found to act as carriers of as much as 40% of the total vitamin A added to milk. Whereas earlier studies indicated the ability of reassembled casein micelles to act as nanocarriers, nanovehicles or microencapsulates, the present study further shows the ability of unmodified casein micelles to bind hydrophobic molecules such as vitamin A.

Acknowledgements
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References


Official Methods of Analysis. 2006. Method 2002.06 Retinyl palmitate (Vitamin A) in fortified fluid milk. 18th ed. AOAC international, Gaithersburg, MD.


Appendix

Figure 3A.1: FPLC chromatograph of milk-1 sample along with SDS-PAGE gel fragments indicating proteins present in each peak of the chromatograph. Fractions obtained by FPLC pooled together as sections (1 to 8).
Figure 3A.2: FPLC chromatograph of milk-2 sample along with SDS-PAGE gel fragments indicating proteins present in each peak of the chromatograph. Fractions obtained by FPLC pooled together as sections (1 to 6).
Figure 3A.3: FPLC chromatograph of milk-3 sample along with SDS-PAGE gel fragments indicating proteins present in each peak of the chromatograph. Fractions obtained by FPLC pooled together as sections (1 to 8). The gel fragment for Section 3 is missing.
Figure 3A.4: FPLC chromatograph of milk-4 sample along with SDS-PAGE gel fragments indicating proteins present in each peak of the chromatograph. Fractions obtained by FPLC pooled together as sections (1 to 7).
Table 3A.1: Percentage peak areas within total peak area of all proteins (Peak 1+Peak 2+Peak 3+Peak 4) in the FPLC chromatographs of different skim milk samples.

| Samples | Percentage peak Area¹ | | | | | |
|---------|-----------------------|------------------|------------------|------------------|------------------|
|         | Peak 1    | Peak 2    | Peak 3    | Peak 4    |
|         | Mean      | SE        | Mean      | SE        | Mean      | SE        | Mean      | SE        |
| Milk-1  | 81.1      | 1.0       | 6.2       | 0.82      | 6.9       | 0.23      | 5.7       | 0.40      |
| Milk-2  | 86.3      | 9.1       | 3.7       |           |           |           |           |           |
| Milk-3  | 82.4      | 4.9       | 6.6       |           |           |           |           |           |
| Milk-4  | 88.6      | 3.5       |           |           | 2.6       |           |           |           |

¹Peak area represented as percentage of total peak area of the protein peaks in that skim milk sample. Mean of duplicates for each skim milk.

a–b Values within a column followed by a different superscript differ (P < 0.01).
Table 3A.2: Retinyl palmitate content in skim milks and casein section of skim milks along with retention of retinyl palmitate content in casein sections as a percentage of the total content in each skim milk sample.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Retinyl palmitate content</th>
<th>Percentage retention of retinyl palmitate in casein section</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mL milk</td>
<td>µg/mL milk</td>
</tr>
<tr>
<td>Milk-1</td>
<td>2.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.918&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milk-2</td>
<td>1.59&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.222&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milk-3</td>
<td>2.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.555&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milk-4</td>
<td>1.82&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.734&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean calculated for duplicates for each skim milk sample.

<sup>a-d</sup>Values within a column followed by a different superscript differ (P < 0.05).
CHAPTER 3B
STABILITY OF VITAMIN A IN RAW SKIM MILK AND APPLE JUICE
ON EXPOSURE TO ULTRAVIOLET LIGHT
Vitamin A palmitate is enriched in commercial milk, where earlier studies indicated that associates with casein micelles. We investigated whether casein micelles protect vitamin A palmitate from degradation on exposure to ultraviolet (UV) radiation. Raw skim milk and apple juice were fortified with vitamin A palmitate, and then subjected to strong UV radiation for diverse time periods. Similarly, samples of milk fortified with vitamin A was subjected to size exclusion chromatography (SEC) and the eluted fractions containing vitamin A were pooled, freeze dried and analyzed for vitamin A content. On exposure to very high UV light intensity (880 µWcm$^{-2}$), the vitamin A in milk and juice degraded at the same rate and were reduced to about 6% of the initial quantity after 60 min of exposure. With the reduction of UV intensity to 132 µWcm$^{-2}$, the degradation pattern for vitamin A in milk and juice was varied with rate of degradation of 4.4 and 7.3%/min in the first 10 min of exposure, respectively. Further reduction of UV intensity to 53 µWcm$^{-2}$ resulted in a rate of degradation of 0.3 and 3%/min, respectively. There was rapid degradation of vitamin A palmitate in juice samples, with a reduction of 59% vitamin A of the initial amount added on exposure for 20 min to UV light (53 µWcm$^{-2}$). While 60% of vitamin A in juice was degraded upon exposure for 20 min to low intensity UV light (53 µWcm$^{-2}$), only 6% was degraded in milk samples. Quantification of vitamin A in the SEC fractions indicated that vitamin A palmitate was linked only to casein micelles in milk, with a recovery of 31% of the initial amount added within the casein section. The results indicate that
coupling of vitamin A palmitate with casein micelles in raw skim milk protects vitamin A palmitate against degradation on exposure to UV light.

**Introduction**

The milk system as designed by nature as an effective transport media for nutrients from the mother to the neonate. The caseins in milk form a complex protein assembly called casein micelles. These micelles transport large amounts of calcium (1180 mg/kg of milk), phosphorus (1030 mg/kg of milk) and proteins in an assimilative form without precipitation or separation. Milk is also a good source of vitamin A (0.18µg/mL milk), among other nutrients (USDA-ARS, 2011). Since vitamin A is fat soluble, is normally associated with the milk fat globules, explaining why vitamin A is absent when fat is removed from milk during processing. This process is the rationale for fortification commercial milk with vitamin A, usually in the form of retinyl palmitate.

Retinyl palmitate is a thermally stable ester of retinol and is the primary vitamin A storage form in the body (Brinkmann et al., 1995, Tolleson et al., 2005). Retinyl palmitate has a β-ionone core and a chain which provides an electron dense region that is attracted to radicals and other electron deficient species. Thus it undergoes a number of reactions on subjecting to different conditions including exposure to light, transition metals, oxygen and thermal treatment (Loveday and Singh, 2008). Retinoids undergo a number of photochemical reactions by different mechanisms including photoisomerization, photopolymerization, photooxidation and photodegradation (Dillon et al., 1996, Mousseron-Canet, 1971, Mousseron-Canet et al., 1966, Tolleson et al., 2005). Depending on the time of exposure and wavelength of light, retinyl palmitate undergoes photoisomerization to form a mixture of *trans* and *cis* isomers, mainly 9 *cis*
and 13 cis isomers (DeMan, 1981, Gaylord et al., 1986a, Jung et al., 1998, Murphy et al., 1988). Photooxidation of vitamin A is complex, involving a number of products that can undergo further oxidation (Crank and Pardijanto, 1995, Failloux et al., 2004). The photooxidation of retinyl palmitate in ethanol irradiated using UVC (254nm) light produced anhydroretinol, palmitic acid and 2-buteny palmitate (Crank and Pardijanto, 1995). In another study when retinyl palmitate in methanol was exposed to UVA (365 nm) for 15 min products such as palmitic acid, anhydroretinol, 4,5-dihydro-5-methoxy-anhydroretinol and a dihydro-methoxy-anhydroretinol isomer with three conjugated double bonds were detected (Tatariunas and Matsumoto, 2000). Cherng et al.(2005) reported that photolysis of retinyl palmitate (dissolved in ethanol) on exposure to UV light (14 J/cm²; 98.9% UVA (315 - 400nm), 1.1% UVB (280 – 315 nm) and <0.0001% UVC (250-280nm) secured 14 degradation products. Similar degradation products were observed on exposure to UVA light by another group (Xia et al., 2006).

Since many of the aforementioned studies were conducted with retinyl palmitate naturally present, it becomes especially important to understand the effects of light (visible and UV), oxygen and temperature on retinyl palmitate when added to food products, as these conditions contribute to the storage of food. Such studies are, however, scarce. Kim et al. (2000) reported substantial loss of retinyl palmitate fortified in corn flakes on storage at ambient temperature for 16 weeks. The degradation pattern of retinyl palmitate is highly dependent on other pro-oxidant (like free radicals form unsaturated fatty acids) and anti-oxidant (including some amino acids and vitamins) factors existing within the food system (Halbaut et al., 1997, Kim et al., 2000). There are only few studies on the effect of light and oxygen on vitamin A in milk and milk products. Some report the isomerization of retinyl palmitate in milk stored under retail
fluorescent lighting conditions (Gaylord et al., 1986a, Jung et al., 1998, Murphy et al., 1988). Others observed that photooxidation products of retinyl palmitate enriched in nonfat dry milk and pasteurized low fat milk produce hay like off-flavor on storage (Nakai et al., 1983, Suyama et al., 1983). Although these studies have been conducted on the loss of vitamin A from milk and milk powder, the rate of degradation of retinyl palmitate added to milk has not been compared to that added to other food systems.

There have been many studies on the hydrophobic interactions within casein micelles. These interactions contribute to their ability to associate with a number of hydrophobic compounds including vitamin A, docosahexanoic acid, green tea flavanoids and curcumin (Mohan et al., 2013, Pan et al., 2013, Yuksel et al., 2010, Zimet et al., 2011). Many of these studies were conducted using re-assembled casein micelles. In contrast, casein micelles in their native conditions have been observed to have water filled channels and big cavities (Trejo et al., 2011), which enable the micelles to entrap very small hydrophobic compounds. The other study conducted in our laboratory indicated that casein micelles in their native state associate with vitamin A in commercial pasteurized fortified skim milk (Chapter 3A). Therefore, we hypothesized that the association of retinyl palmitate to casein micelles could provide protection from photodegradation. This hypothesis is consistent with other studies reporting that samples of milk containing inherent and fortified vitamin A showed faster degradation rate for the fortified retinyl palmitate compared to the degradation of the inherent vitamin A in milk, on exposure to sunlight and fluorescent light (DeMan, 1981, Jung et al., 1998, Thompson and Erdody, 1974). Hence, our objective was to compare the stability of vitamin A (retinyl palmitate) in raw skim milk and apple juice.
Materials and methods

Sample preparation
Raw milk samples were collected from the UT Little River experimental farm. It was skimmed by centrifugation at 6414 × g for 20 min and manually removed the top fat layer using a spatula. This procedure was repeated until no visible layer of fat existed. Apple juice was obtained from 1cm thick slices of Gala apples by steam blanching them until soft (for 10 to 12 min using a Steam blancher, Hobart Corporation, Troy, Ohio), and manually squeezing the juice out after wrapping the slices in cheese cloth. The apple juice was then strained and stored at -20°C until analysis. Vitamin A palmitate (approx. 15 mg, Sigma-Aldrich, St. Louis, MO) was weighed in to glass centrifuge tube, mixed with 0.5 mL of 100% ethanol and vortexed until dissolved. This solution was then mixed with 20 mL of raw skim milk or apple juice and then vortexed for 30 sec. The sample prepared was then rotary homogenized at 10,000 rpm for 3 min (Polytron PT 10-35 GT, Kinematica AG, Lucerne, Switzerland). About 5mL of the prepared sample was added to two scintillation vials (Fisher Scientific, Pittsburg, PA). Milks and juice vials in duplicates were placed in random order on a tube rotator as shown in Figure 3B.1. A UV transilluminator (wavelength 365nm, Model TLW-20,UVP, LLC, Upland, CA) with a surface UV light power of the lamp 5300µW/cm² (as declared by manufacturer) was used. The tube rotator with the samples was placed at distances 6, 40 and 100 cm away from the UV light source in a walk in cooler at 4°C. The following equations were used to calculate the cumulative UV intensity exposure: \[ P_R = \frac{P_L}{S} \] and \[ D = P_R \times t, \] where \[ D = \text{Cumulative UV intensity exposure (J/cm}^2) \], \[ P_L = \text{Surface UV light power of the lamp as declared by manufacturer (W)} \], \[ P_R = \text{UV light intensity or UV light power of radiation per unit surface area (W/cm}^2) \], \[ t = \text{Time of exposure to irradiation (sec)} \], \[ S = \text{Lateral surface of the emission cylinder = Length of the lamp ×} \]
distance of sample from light source. When the samples were placed at 6 cm from the UV transilluminator, the samples were extracted for vitamin A content every 15 min during an hour (0, 15, 30, 45 and 60 min). When the samples were at distances 40 and 100 cm away from the UV source, the time period of study was every 5 min for 20 min (0, 5, 10, 15 and 20 min). The distance along with time period of exposure was adjusted to optimize the degradation pattern of vitamin A in the samples. After each time period 1 mL of the sample was removed from each vial and vitamin A extracted. The sample left in the vial was replaced on the tube rotator for further UV exposure. The absorbance values of milk and juice was measured using a UV spectrophotometer (Helios Biomate 5, Thermo electron Corp., UK) at 365 nm wavelength by diluting the samples using de-ionized water to a concentration of 5% v/v.

**Size exclusion Chromatography**
Three replicates of vitamin A milk samples were prepared using the procedure mentioned earlier. The samples were sequentially subjected to size exclusion chromatography (SEC); while a sample was analyzed, the other samples were stored at 4°C (three replications). The samples (2 mL) were separated into protein fractions using an SEC column (HiLoad 16/60 Superdex 200 prep grade, GE Life Sciences, GE Health Care, Piscataway, NJ) connected to a fast protein liquid chromatography (FPLC) unit (AKTA, GE Life Sciences). Ice-cold protein-free serum obtained by ultrafiltration (cellulose tangential-flow ultrafiltration system with a molecular weight cutoff of 3 kDa, PLBC Prep scale TFF Cartridge, Millipore, Billerica, MA) of pasteurized skim milk and preserved with 0.07% (w/v) sodium azide at 4°C, was used as the mobile phase. Separation of proteins in the sample was achieved using a flow rate of 1.0 mL/min for 180 mL, collecting 1-mL fractions using an automatic fraction collector (Frac-950, GE Life Sciences). Elution of
protein peaks was detected using UV absorbance at 280 nm. Collected fractions within the same peak (protein milk sections) were pooled and freeze-dried.

**HPLC**

Extraction of vitamin A from skim milk, juice and freeze dried samples was done by liquid-liquid extraction process modified from the AOAC method 2002.06 (Official Methods of Analysis, 2006), as detailed previously (Mohan et al., 2013). Briefly, 5 mL of 100% ethanol was added to 1 mL milk samples in a 15 mL centrifuge tube, vortexed for 30 sec and allowed to stand for 5 min. Then 2.5 mL hexane were added to it, vortexed for 30 sec and allowed to stand for 2 min. The samples were vortexed and allowed to stand, repeatedly three times. Finally, 1.5 mL DI water were added, vortexed for 5 sec (no longer), and centrifuged for 10 min at 1800 rpm (633 x g) using a Sorvall RC-5B super speed centrifuge with SH-3000 Aluminum rotor. The top hexane layer (100 µL) was auto-injected in to an HPLC system (1200 series, Agilent) with a normal phase Zorbax RX Sil 4.6 x 250 mm (Agilent #880975-901) column set at 23°C. The conditioned hexane prepared from 99.85% v/v of wet hexane (500 mL hexane conditioned with 25 mL distilled water overnight) and 0.15% v/v of Isopropanol was used as mobile phase pumped at a flow rate of 1mL/min. The vitamin A (retinyl palmitate) peak was detected between 2.5 and 3.5 min of retention using 325 nm wavelength by the Diode Array detector. The vitamin A content of samples exposed to UV light was represented as a percentage of the initial vitamin A content in the sample at the beginning of the test (0 min). The vitamin A content of the milk casein FPLC fractions was calculated using a linear standard curve (y = 6721.6x + 11613), employing a retinyl palmitate standard (#R1512, Sigma-Aldrich, St. Louis, MO) dispersed in a similar manner as the samples in milk and expressed as a percentage of the initial quantity added in milk. The freeze-
dried sections were diluted with 1 mL distilled water and extracted with proportionate modifications in the amount of extraction solvents by the same method. In order to avoid the effect of storage conditions on the retinyl palmitate detected in the present study, the vitamin content in the milk after subjecting to deterioration conditions for specific time intervals was expressed as a percentage of the amount detected at ‘0’ time. The rate of degradation for linear regions of the degradation curve was obtained from the slope of the best fitting linear model for the linear section of the curve considered by trial and error to produce an $R^2$ value of greater than 0.85. This was represented as the change in the percentage of retained retinyl palmitate in the sample within unit time of exposure (%/min).

**Statistical analysis**
The mean and standard error of the replications for the different treatments (6 cm; 4 replications, 40 cm:2 replications and 1 m:4 replications) was calculated by ANOVA using the Mixed procedure in SAS 9.2 (SAS Institute). The mean separations were considered significant for $P < 0.05$ using Least Significant Difference method.

**Results and discussion**

**Size exclusion chromatography**
The size exclusion chromatography (SEC) of milk containing retinyl palmitate yielded different fractions containing proteins. The freeze dried powder from the casein micelle section (section 1, as in Chapter 3A) contained about $31 \pm 2\%$ of the retinyl palmitate added to the milk samples. Although very small retinyl palmitate peaks were detected in all other fractions by HPLC, the area under these peaks was negligible and unable to be quantified. These results indicate the association of retinyl palmitate in the milk samples was mainly with casein micelles. This is
consistent with the observations from our previous study indicating the association of retinyl palmitate with casein micelles when fortified commercially in pasteurized skim milk. The study also indicated retention of 14 to 40% of retinyl palmitate in association with the casein micelles in milk (in Chapter 3A). Many studies indicated the ability of reassembled casein micelles to interact with hydrophobic probes like triclosan, vitamin D₂, docosahexanoic acid, curcumin, mitoxantrone, green tea flavanoids (Pan et al., 2013, Semo et al., 2003, Shapira et al., 2010, Yuksel et al., 2010, Zimet et al., 2011). Efforts also have been made to study binding capacities of casein micelles under natural conditions. In one such study, Roach and coworkers attempted to isolate casein micelles from milk by ultracentrifugation and mixed triclosan into re-dispersed casein micelles using ethanol solvent (Roach et al., 2009). They observed 75% association of triclosan to casein micelles. Further, our retinyl palmitate quantification in casein micelle fractions from fortified milk indicate natural association between casein micelles and added retinyl palmitate dispersed by rotary homogenization in the presence of very small quantities of ethanol.

As can be seen in Figure 3B.1, the retinyl palmitate peak in the HPLC chromatogram decreased with increasing time elapsed before SEC. Considering the area under the peak at time ‘0’ to be 100%, the peak area reduced to 74.9% of the initial peak area (at ‘0’ time) after 2 h. A further reduction to 48.3% of the initial peak area was observed after 4 h of preparation of vitamin milk sample. Although this analysis was not replicated, many previous researchers have reported oxidative degradation of vitamin A (Allwood and Martin, 2000, Gaylord et al., 1986a, Jung et al., 1998, Kim et al., 2000, Murphy et al., 1988, Nakai et al., 1983). They indicated the presence of light and oxygen to greatly enhance the degradation of retinyl palmitate (Nakai et al., 1983,
Sattar et al., 1977, Thompson and Erdody, 1974) and even formation of hay-like off flavor in commercially available vitamin A fortified fluid milk (Suyama et al., 1983). The HPLC chromatography did not indicate the presence of other isomers apart from the all-
trans retinyl palmitate, even after degradation, implying the chances of photo-oxidation as the mechanism of degradation as opposed to photo-isomerization. In the present study, the samples prepared at ‘0’ time were capped and stored under refrigeration until SEC. Thus, oxygen within the vials and the initiation of oxidation in the presence of light was partially responsible for degradation. Moreover, the milk fractions collected during SEC of vitamin milks were kept exposed to light and O₂ for about 2 h during the entire run, which may be one of the reasons for the retention of only up to 31% of the retinyl palmitate in the casein micelle section from replicates of vitamin milk samples, as mentioned earlier. Similar low retention of retinyl palmitate in fortified commercial pasteurized milk has also been reported (Mohan et al., 2013). Another study conducted by Semo et al. (2003) accounted for only 85% of vitamin D initially added as being associated with the casein micelles. On the whole, the conditions of testing and storage need to be properly controlled to enable better retention of retinyl palmitate in such studies. However, it becomes almost impossible to eliminate the effect of storage conditions affecting light sensitive compound analysis, especially for time consuming procedures like SEC.

**Exposure to UV light**

At a distance of 6 cm between the sample and the UV light source (UV light intensity of 883.3 µWcm⁻²), retinyl palmitate in both milk and juice samples deteriorated rapidly to about 10% of the initial quantity after 30 min of UV exposure (Figure 3B.2). The rate of degradation for the first 30 min for milk and juice was 5.6%/min (Table 3B.1), and was reduced to 0.5%/min on UV
exposure for 40 to 60 min. A similar reduction in the rate of degradation and existence of a
minimum degradation concentration (below which retinyl palmitate does not deteriorate), has
been reported previously (Bartholomew and Ogden, 1990, Thompson and Erdody, 1974). Our
results also indicate that at a very high dose of UV exposure, the retinyl palmitate degrades
rapidly regardless of the system in which it has been dispersed.

A reduction of UV exposure intensity by increasing the distance of samples from the UV source
to 40 cm (UV light intensity of 132.5 µWcm⁻²; Figure 3B.3) resulted in 100, 84, 57, 30, and 16
± 6% of retinyl palmitate retained in the milk samples on exposure to UV light for 0, 5, 10, 15,
and 20 min, respectively. The degradation pattern of retinyl palmitate content indicated that there
were 100, 64, 27, 11, and 10 ± 6% retinyl palmitate retained in the juice samples on exposure to
UV light for 0, 5, 10, 15, and 20 min, respectively. The amount of retinyl palmitate retained in
the milk sample was significantly higher as compared to that in juice samples after exposure to
UV light for 5, 10 and 15 min time periods. This indicates the ability of milk to protect or
stabilize retinyl palmitate on exposure to UV light. However, the amount of retinyl palmitate
retained in milk was not different from that of juice after 20 min of UV light exposure. The rate
of degradation of retinyl palmitate in milk showed a linear trend throughout the 20 min of
exposure to UV light (4.4%/min; Table 3B.1). In contrast, the rate of degradation in juice did not
fit a single linear model throughout the entire exposure time for 20 min (two linear models with
rate of degradation: 7.3%/min for 0 to 10 min and 0.34%/min for 15 to 20 min). The rate of
degradation of retinyl palmitate in juice became much slower after 15 min, indicating that it
degrades much faster in juice and reaches the minimum degradation concentration after 15 min.
The UV light exposure intensity was further decreased to study the linear region of the degradation curve by increasing the distance between the sample and the UV light source to 1 m (UV light intensity of 53 µWcm⁻²). Retinyl palmitate content obtained was 100, 100, 99, 97, and 94 ± 4% of the retinyl palmitate retained in the milk samples after exposure to UV light for 0, 5, 10, 15, and 20 min, respectively (Figure 3B.4). While the degradation pattern of the retinyl palmitate indicated 100, 97, 88, 67, and 41 ± 4% of that retained in the juice samples on exposure to UV light for 0, 5, 10, 15, and 20 min, respectively. There was a rapid increase in the rate of degradation of retinyl palmitate, especially in apple juice after 10 min of UV exposure. The degradation pattern of milk and juice attained a linear model with $R^2 > 0.85$ on exposure to UV light intensity of 53 µWcm⁻². The rate of degradation of retinyl palmitate was much lower in milk (0.33%/min) compared to that in juice (2.97%/min; Table 3B.1). This further demonstrates that milk provides protection to retinyl palmitate against UV light initiated degradation, which is much greater than that provided by apple juice samples.

The protection against UV light triggered photo-oxidation of retinyl palmitate in milk samples was associated with many factors. It is well known that proteins absorb UV light at 365 nm wavelength as indicated by its absorbance of 1.2 A. Consequently, the absorbance of UV light by proteins contribute to the protection of retinyl palmitate provided by milk with approx. 3.5% protein content as compared to negligible amount of proteins present in apple juice. The very low protein content of apple juice is also indicated by the absorbance value of 0.1 A for apple juice at UV light wave length of 365 nm. The major constituent of apple juice (about 10%) is sugars, which contribute very less to absorbance of UV light. It has been reported previously that milk with 1% added milk solids not fat (MSNF) did not provide any additional protection against
degradation to retinyl palmitate on exposure to fluorescent light as compared to normal milk. However in the same study, increases in the amount of added MSNF to 3% provided some protection to retinyl palmitate on exposure to fluorescent light (Gaylord et al., 1986b). Further studies need to be conducted to ensure the ability of casein micelles to provide protection to retinyl palmitate on account of its association with it and not based on the masking ability of proteins to UV light.

Another anomaly that needs to be further elucidated is the nature of association of casein micelles with retinyl palmitate. Many previous studies have indicated that casein micelles in milk associate with many hydrophobic probes owing to their characteristic structure and hydrophobic forces stabilizing it (Livney, 2010). In the present study, about 31% of the retinyl palmitate added to milk associated with casein micelles in the milk samples. Thus the chances are that casein micelles are the major contributing factor to the stability of retinyl palmitate against degradation on exposure to UV light. However, there are no previous studies on the protective ability of casein micelles on any hydrophobic probe associated with it. Hence the stability of retinyl palmitate on UV exposure has been explained based on its association with casein micelles that protects it from photo-oxidation. The precise mechanism of protection of retinyl palmitate can be deciphered only by further understanding the type of association of casein micelles with retinyl palmitate.

**Conclusions**

Our observations indicate that retinyl palmitate added to milk samples is protected from UV light degradation compared to apple juice. This protection was associated with the hydrophobic binding properties of casein micelles to retinyl palmitate owing to its hydrophobic nature and
small size. An item of concern that requires further investigation is the intrinsic absorbance of UV light by proteins that could also mask the retinyl palmitate from direct exposure. In order to further understand the ability of casein micelles to protect against photodegradation of retinyl palmitate we need to elucidate the nature of the association of these molecules with the casein micelles.

**Acknowledgements**

We would like to acknowledge the Little River Animal facility associated with the University of Tennessee for providing the raw milk required for the study.
References


Official Methods of Analysis. 2006. Method 2002.06 Retinyl palmitate (Vitamin A) in fortified fluid milk. 18th ed. AOAC international, Gaithersburg, MD.


## Appendix

Table 3B.1: Rate of degradation of retinyl palmitate added to milk and juice on exposure to UV light for varying time periods adjusting the UV light intensity by adjusting the distance of the samples from the UV source

<table>
<thead>
<tr>
<th>Distance of sample from UV light and UV light intensity</th>
<th>Time (min)</th>
<th>Milk Rate of degradation (%/min)</th>
<th>Milk $R^2$</th>
<th>Juice Rate of degradation (%/min)</th>
<th>Juice $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 cm (883.3 µWcm$^{-2}$)</td>
<td>0</td>
<td>5.6</td>
<td>1</td>
<td>5.6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.6</td>
<td>1</td>
<td>5.6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.15</td>
<td>1</td>
<td>0.15</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.15</td>
<td>1</td>
<td>0.15</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.15</td>
<td>1</td>
<td>0.15</td>
<td>1</td>
</tr>
<tr>
<td>40 cm (132.5 µWcm$^{-2}$)</td>
<td>0</td>
<td>4.4</td>
<td>0.99</td>
<td>7.3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.4</td>
<td>0.99</td>
<td>7.3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.4</td>
<td>0.99</td>
<td>7.3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4.4</td>
<td>0.99</td>
<td>0.34</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.4</td>
<td>0.99</td>
<td>0.34</td>
<td>1</td>
</tr>
<tr>
<td>1 m (53 µWcm$^{-2}$)</td>
<td>0</td>
<td>0.33</td>
<td>0.87</td>
<td>2.97</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.33</td>
<td>0.87</td>
<td>2.97</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.33</td>
<td>0.87</td>
<td>2.97</td>
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</tr>
<tr>
<td></td>
<td>15</td>
<td>0.33</td>
<td>0.87</td>
<td>2.97</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.33</td>
<td>0.87</td>
<td>2.97</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Figure 3B.1: HPLC chromatogram for the peaks of retinyl palmitate in the casein micelles obtained from size exclusion chromatography for three replicates of milk samples subjected to SEC 0, 2 and 4 hrs after homogenization.
Figure 3B.2: Degradation pattern of retinyl palmitate in milk and juice on exposure to UV light intensity of 833.3 µW cm\(^{-2}\). Standard error for milk and juice values is 1%. 
Figure 3B.3: Degradation pattern of retinyl palmitate in milk and juice on exposure to UV light intensity of 132.5 µW cm\(^{-2}\). Error bars indicate the standard error for milk and juice values.
Figure 3B.4: Degradation pattern of retinyl palmitate in milk and juice on exposure to UV light intensity of 53 µWcm\(^{-2}\). Error bars indicate the standard error for milk and juice values.
CHAPTER 4A
EFFECT OF ULTRA-HIGH PRESSURE HOMOGENIZATION ON PHYSICOCHEMICAL PROPERTIES OF PASTEURIZED SKIM MILK
This chapter is a lightly revised version of a paper by the same title to be submitted to the Journal of Dairy Science by M. S. Mohan, R. Ye, L. Liu and F. Harte. The use of “our” in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the sample preparation, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the manuscript writing.

Abstract
Developments in material science and engineering have enabled high pressure homogenization (HPH) at >500 MPa processing pressure. The objective of this study was to characterize the physicochemical properties of pasteurized skim milk homogenized at 0 to 500 MPa to explore novel uses of milk and milk ingredients. We studied the effect of HPH on pH, apparent casein micelle size (dynamic light scattering; pH 2 to 10), turbidity (absorbance at 550 nm; pH 2 to10), shelf stability at 4ºC, heat stability (120ºC; pH 6.3 to 6.7), ethanol stability (% required to coagulate; pH 6.1-7.1), viscosity (flow curve), and viscoelasticity after rennet addition (G’; 90 min, 1 Hz, 0.1% strain). The HPH milks had a maximum of 0.13 pH units higher than milk not subjected to HPH. The apparent particle size increased from ca. 180 nm in 100 MPa milk to ca.280 nm in 500 MPa milk. There was good positive correlation of particle size data with absorbance data (r =0.63, P<0.05). Despite the increase in particle size of casein micelles, all milk samples were shelf stable up to 14 days at 4 ºC and all milks coagulated when adjusted to 4.5 pH. At pH 5, the HPH processing of milk promoted early aggregation, as micelles went from ca. 250 nm at 0 MPa to ca. 300 nm and ca. 500 nm after processing at 400 and 500 MPa, respectively. From pH 6 to 8 the casein micelle size of 500 MPa milk decreased until it was same at pH 8 to10 with 0 MPa milk. Although a higher absorbance of 0 MPa than 500 MPa milk at pH 8 to 10 was observed indicating differences in the observation of particle size and absorbance, the trend was similar for both measurements at pH 6 and 7. The 0 and 500 MPa milks coagulated at 6.4 pH in 12 min and 6.5 pH in 24 min on heat treatment at120ºC, respectively. All the milks
coagulated on heat treatment at 120°C for 3 min at pH 6.3. Ethanol stability decreased with increasing pressure of HPH at all pH. The viscosities of 400 (ca. 4.1 mPa.s) and 500 MPa (ca. 4.2 mPa.s) milk were higher than 0 MPa milk (ca. 2.2 mPa.s). With increase in HPH pressure renneting ability decreased until no coagulation was observed for milk processed at 500 MPa. Hence, 500 MPa pressure altered the pH, viscosity and response of milk to various pH, heat, ethanol and renneting conditions. Therefore, the present study enables the utilization of ultra-high pressure homogenization as a technique to modify the structural and physicochemical characteristics of casein micelles in pasteurized skim milk.

**Introduction**

In the past couple of decades the food processing industry has become increasingly aware of certain advantages of high pressure over thermal treatments for processing of foods. Unlike heat transfer, pressure is transmitted uniformly in a pressure chamber to all particles. High hydrostatic pressure (HHP) is becoming a preferable nonthermal processing technique for inactivating harmful vegetative pathogens, spoilage microorganisms and enzymes in different food products. However, HHP is a batch processing operation that has limited use in the case of liquid and semisolid food products where continuous processes are preferred. This brings homogenization to the forefront as a continuous processing operation consisting of a positive displacement pump which pushes the fluid through a homogenizing valve (Middelberg, 1995). High pressure homogenization (HPH) systems used previously enabled pressure treatments up to 400 MPa pressure. The most common HPH systems produce high homogenization pressures with the help of zirconium or tungsten carbide needle-seat or ball-seat valves (e.g., Stansted Power Fluid, Harlow, UK) and diamond, sapphire or ruby nozzle (e.g. Avestin, Inc., Ottawa, Canada, BEE
international, Massachusetts, USA). During HPH, a combination of other physical phenomena including hydrostatic pressure, impingement, shear stress, turbulence, and cavitation is applied (Paquin, 1999). While, the HHP system applies only hydrostatic pressure to samples in a closed pressure chamber.

In the case of milk and milk components, previous studies demonstrated a reduction of casein micelle size on homogenization of raw skim milk at pressures up to 200 MPa (Roach and Harte, 2008, Sandra and Dalgleish, 2005) and then an increases in size with pressure up to 350 MPa (Roach and Harte, 2008). The reduction in particle size at pressure of 100 to 200 MPa was associated with the partial or complete dissociation of the casein micelles and migration of κ and α_{s1} caseins to the serum phase (Hayes et al., 2005, Roach and Harte, 2008, Sandra and Dalgleish, 2005). The increase in the micelle size from 200 to 350 MPa was explained by an increase in hydrophobic interactions among individual caseins due to the shear-induced higher temperature in the homogenization valve (Roach & Harte, 2008). The increase in casein micelle size has also been suggested to occur by the formation of complexes of denatured β-Lactoglobulin (β-Lg) and caseins (Escobar et al., 2011, Hayes et al., 2005, Zobrist et al., 2005), and micellar aggregation after the removal or collapse of the κ-casein brush layer on the micelle surface (Needs et al., 2000) in skim milk subjected to HPH and HHP treatments.

There are also other factors affecting the changes in the casein micelles size in HPH milks. Temperature is a major factor affecting the size of the casein micelles during HHP treatment and subsequent storage (Needs et al., 2000). A reduction in casein micelle size and turbidity (measured as absorbance at 600 nm) of pasteurized skim milk at 55°C after homogenization at 100, 200 and 300 MPa followed by cooling to 4°C was reported by Lodaite et al. (2009).
Variations have been observed in the pattern of casein micelle size increase or reduction with application of HPH by different research groups (Lodaite et al., 2009, Roach and Harte, 2008). It has been associated with variations in the hydrophobic interactions induced by the temperature profile before and after the high pressure treatment.

A number of investigators have also studied other properties of HPH milks including gelation by acids and enzyme. Many of the HPH studies mentioned have been conducted on raw and pasteurized whole milk (Hayes et al., 2005, Lee et al., 2009, Thiebaud et al., 2003). There are differences in the behavior of the milk system without fat (skim milk) and with fat (whole milk).

This was demonstrated by Lodaite et al. (2009) in their study of rheology of rennet gels of skim milk and milk standardized with 3.4% fat. They observed a decrease in gelling time and increase in G’ in the skim milk rennet coagulated gels on increasing the HPH pressure to 300 MPa. This has been associated with the decrease in the size of casein micelles and the higher homogeneity of the gel network formed by HPH milks. The standardized milks also exhibit a lower gelling time and higher G’ values at higher homogenization pressures, which has been associated with the increase in the number of interacting moieties including casein micelles and small fat globules. In many of these studies the gelling time of rennet gels were directly correlated with the casein micelles size at different pressures (Lodaite et al., 2009, Sandra and Dalgleish, 2007).

According to Lodaite et al. (2009), the correlation between reduction in gelling time and the reduction in casein micelles size has been explained by perikinetic aggregation where smaller particles gain greater mobility due to Brownian motion, collide with each other and stick together to form gel (Walstra, 2003). The smaller casein micelles form stiff rennet gels with higher G’ values. However, there are other studies that report increase in gelling time with
increase in homogenization pressure. They have associated the increase in rennet coagulation
time of whole milk at high pressure to the higher availability of κ-casein in solution and lower
critical level for chymosin action, steric hindrance due to incorporation of β-Lg along with casein
micelles on the surface of fat globules, increase in pH, and reduction in the soluble ionic calcium
(Zamora et al., 2007). These varied explanations and phenomena are an indication of the
complex association of the different interaction forces and molecules in HPH milk.
The recent developments in material science and engineering have enabled the development of
pumps able to deliver dynamic pressures up to 600 MPa. These devices (also called waterjets)
are used to cut tough or brittle materials (e.g., metals, granite, marble) using a pressurized stream
of water and abrasives. The pressure in these systems is developed by an intensifier pump,
maintained using an attenuator cylinder and the fluid pushed through a small orifice (1 to 10 µm)
in a diamond, ruby, or sapphire nozzle. This pump system also acts as an ultra-HPH system
where all the dynamic pressure forces including hydrostatic pressure, impingement, shear stress,
turbulence, and cavitation are generated. In the present study we investigate the effect of ultra-
HPH (up to 500 MPa) on the physicochemical properties of pasteurized skim milk (viscosity,
heat stability, ethanol stability and pH stability), along with casein microstructure and
rheological properties of rennet gels made from ultra-HPH milk.

Materials and methods

Ultra-high pressure homogenization
Pasteurized skim milk was bought at a local grocery store. The skim milk was subjected to
homogenization from 0 to 500 MPa in 100 MPa increments using a Hyperjet pump system (Flow
International Corporation, Kent, WA). The pressure was maintained by an intensifier pump and
sprayed through a 4 µm pore size diamond nozzle. The spray of HPH milk from the nozzle was contained in a tubular heat exchanger (concentric tubes with outer tube diameter of 3 inches and inner tube diameter of 2 inches with a height of 4 feet). An aqueous glycol solution was pumped from a water bath set at -32ºC to cool down the homogenized milk. The temperature of the HPH milk samples and the refrigerant were constantly monitored (Figure 4A.1). The pump was cleaned with DI water and a neutral detergent (Softclean No. 379, Hydrite, Brookfield, WI), after running all the pressures for each replication and then rinsed with DI water. The homogenized milk samples were stored in a cooler set to 4ºC until analysis. All the analysis of the milk samples were conducted within three days of the homogenization process.

**SDS-PAGE**

The SDS-PAGE was performed adapting a published method (Laemmli, 1970) on HPH milk and supernatant obtained by ultracentrifugation (Beckman Coulter Optima L-100K ultracentrifuge) in polycarbonate tubes (Beckman Coulter, Inc., Fuller, CA) at 100,000×g for one hour at 20°C. The HPH milk samples were subjected to SDS-PAGE under non-reducing conditions and the supernatant to reducing conditions. For the supernatant samples subjected to reducing conditions, about 150µL of sample was mixed with 500µL reducing-loading buffer (0.5 M Tris-HCL pH 6.8, glycerol, 10% (w/v) SDS, β-mercaptoethanol, 0.5% (w/v) bromophenol blue in water) and then heat-denatured in a water bath at 95°C for 5 min. For the HPH milk samples subjected to non-reducing conditions, about 150µL of sample was mixed with 500µL non-reducing-loading buffer (0.5 M Tris-HCL pH 6.8, glycerol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue in water). Samples were loaded on an electrophoresis gel (12%Tris-HCl, 12 wells, Ready Gel, BIORAD, Hercules, CA) with 3µL in each well for the non-reducing milk samples and 8 µL in each
well for the reducing supernatant samples. The gel was subjected to a voltage of –200V for 38 min and then stained using coomassie blue stain. The pattern of proteins was identified by comparison to previous research (Jovanovic et al., 2007).

**Particle size**
The particle size analysis was conducted as detailed by Ye and Harte (2013). Briefly, one part of HPH skim milk was diluted with 15 parts of imidazole buffer and then transferred to a dynamic light scattering particle size analyzer (165° angle, 1.34 refractive index, 50-μm pinhole; DelsaNano C, Beckman Coulter Inc., Atlanta, GA). The standards provided by Beckman Coulter Inc. and native casein micelles from raw skim milk diluted with protein free serum (obtained from ultra filtration) were used to calibrate the equipment. The samples were analyzed in a quartz measuring cuvette and controlled for temperature using a water bath (Isotemp 3006S; Fisher Sci., Pittsburg, PA) set at 25°C. The pH of the samples was adjusted using 2N HCl and NaOH and later monitored using a pH electrode (UB-10; Denver Instrument Co., Denver, CO). The apparent particle size of the casein micelles ($D_{3,4}$) were measured as the mean of two replicates calculated from the average of 70 autocorrelation functions.

**Scanning Transmission Electron Microscopy (STEM)**
The homogenized milk sample (5 μL) was placed on a 400 mesh copper grid (glow discharged). After 1 min, the excess sample was removed by blotting from the side of the grid. Then a drop of uranyl acetate was added and allowed to stain for 1 min. The excess stain was removed by blotting with a filter paper. After allowing the grid to dry on a heating block, it was imaged using a Zeiss Auriga electron microscope (Carl Zeiss, Thornwood, NY) in the STEM mode at 30 kV.
**pH profile and pH stability**
The homogenized milk samples were allowed to stabilize to room temperature (25°C) for one hour and pH was measured (pH/ATC combination electrode, Denver Instruments, Bohemia, NY). The 0, 400 and 500 MPa HPH milk samples were adjusted to pH of 5 to 10 (in 1 pH unit increments). The samples were then diluted with 20 mM imidazole buffer at a ratio of 1:15 v/v (skim milk: buffer) and apparent particle size was measured as previously described. The absorbance at 550 nm of pH-adjusted diluted samples was also measured as an indicator of turbidity using a UV-Vis spectrophotometer (Biomate 5, Thermo Fisher Scientific, Waltham, MA).

**Ethanol stability**
The homogenized milk samples were adjusted to pH of 6.1 to 7.1 with 2N NaOH or HCl. Then 2 mL ethanol solution varying from 0 to 100% v/v in increments of 2.5% ethanol was added to 2 mL of milk sample. The mixtures were vortexed and visually examined for protein coagulation. The minimum ethanol concentration required to coagulate the milk proteins was recorded as the ethanol stability (Davies and White, 1958, Horne and Parker, 1981).

**Heat stability**
The homogenized milk samples were adjusted at pH 6.3 to 6.6 and 2 mL aliquots were transferred to autoclavable tubes. Preliminary tests indicated that all milks coagulated at pH 6.3 on heating at 120°C for 3 min. The present study includes results of coagulation of homogenized skim milks on subjecting to heat treatments at 120°C for varying periods of time including 3, 6, 12 and 24 min. The heat treatment was done in autoclaves with a pressure setting of 103.4 kPa. The results were expressed based on visual observation of presence of aggregates and gelation.
Rheology of milks and rennet gels
Newtonian viscosity of processed milks was calculated from the slope of a flow curve (shear rate from 1 to 50 s\(^{-1}\) at 20ºC) measured using a rheometer (AR-2000; TA instruments, New Castle, DE) equipped with a cone and plate geometry (diameter 40 mm, angle 1°, truncation 30 µm). The rennet coagulation of processed milks was done using diluted chymosin enzyme (CHYMAX\(^{®}\) extra, Chr. Hansen Inc., Milwaukee, WI). The test was conducted as recommended by the manufacturer with modification on the final enzyme concentration. A 1:9 dilution of CHYMAX\(^{®}\) extra in DI water was prepared (referred to as CHA) and thoroughly vortexed. Then a 1:9 dilution of CHA in DI water was prepared (referred to as CHB) and thoroughly vortexed. A final 1:4 dilution of CHB in DI water containing 0.2% sodium azide was prepared right before inoculation in milk (referred to as CHC). One mL of CHC solution was added to 100 mL of homogenized milk. Inoculated milks were stirred for 30 sec and then 13 mL transferred to the rheometer (TA AR-2000) equipped with controlled temperature concentric cylinder geometry (15 mm stator diameter, 14 mm rotor diameter, 42 mm height). The samples were subjected to oscillatory testing (G', G", 1 Hz frequency, 0.1% amplitude) over 90 min at 40ºC. A thin layer of low viscosity mineral oil (Cat. No: S55667, Fisher Scientific, Hanover Park, IL) was added to the top of the samples to prevent evaporation during the test.

Statistical analysis
Experiments were analyzed as completely randomized block design with three blocks. Each milk lot subjected to all pressure treatments constituted a block. Statistical evaluation was done using the PROC GLM procedure of SAS 9.2 (SAS Institute Inc., Cary, NC). The particle size, pH stability, gelling time and storage modulus values were calculated as a mean of three replicates. Ethanol stability and temperature profile was studied as a mean of two replications. The means
and mean separations were tested by ANOVA and Tukey-Kramer multiple comparison method. The correlation between absorbance values, apparent casein micelle size and viscosity was analyzed using the Pearson correlation coefficient obtained from CORR procedure in SAS.

**Results and discussion**

*Physical properties of milks after homogenization*

*Temperature profile on HPH.* The temperature of the milks after homogenization and cooling is shown in Figure 4A.1. In spite of the use of the heat exchanger immediately after the homogenization valve, the temperature of the homogenized milk increased from ca. 1°C to ca. 30 °C when homogenization pressure increase from 0 to 500 MPa. There was a sudden increase in temperature from 8°C to 30°C when the homogenization pressure increased from 400 MPa to 500 MPa, respectively. The increase in temperature during homogenization is associated to adiabatic heating due to hydrostatic pressure and shear forces in the homogenization valve (Hayes and Kelly, 2003). Other high pressure homogenization studies have reported ca. 20 °C increase in temperature per 100 MPa increment for high pressure valves not equipped with cooling heat exchangers (Hayes and Kelly, 2003, Pereda et al., 2007, Thiebaud et al., 2003). Since a fixed diamond nozzle homogenization valve (orifice diameter = 4µm) was used in the current study, the flow rate increased from 2.5 to 7.1 mL/sec when homogenization pressure increased from 100 to 500 MPa, respectively. The higher flow rates allowed only lower residence time in the cooling heat exchanger, partially explaining the increment in temperature of HPH milks. The denaturation temperature of the major whey protein, β-Lg is above 70°C (Anema and Li, 2003, Gotham et al., 1992). Although the temperature of the HPH milk was reduced rapidly to below 30°C by the heat exchanger, the temperature of the milk spray soon
after exiting from the high pressure nozzle is not known. Moreover, the combined effect of high temperature, pressure, and shear stress on whey protein denaturation has not been studied extensively (Floury et al., 2000, Paquin, 1999). The SDS-PAGE of milk at non-reducing conditions indicated the gradual reduction in the presence of non-aggregated β-Lg in milks subjected to increasing homogenizing pressures from 100 to 500 MPa as compared to 0 MPa milk (Figure 4A.2A). This indicates the possibility of whey protein denaturation and complex formation within themselves and with caseins by forming disulphide bonds with increase in homogenization pressure (Corredig and Dalgleish, 1996). Similar denaturation of whey proteins promoted by HPH above 200 MPa has been reported in previous studies (Grácia-Juliá et al., 2008, Hinrichs et al., 1996). The SDS-PAGE of supernatant samples at reducing conditions show the composition of the supernatant (Figure 4A.2B). With increase in homogenizing pressure there was a decrease in the amount of caseins in the supernatant samples. This could be associated with the increased removal of aggregates from the supernatant, formed by the association of denatured whey proteins with κ-casein on increasing the homogenization pressure. The complex formation of denatured whey proteins and caseins on high pressure homogenization has been reported earlier (Escobar et al., 2011, Zobrist et al., 2005).

**Apparent casein micelle size.** The mean apparent particle size of the casein micelles in the homogenized milks from 0 to 500 MPa are shown in Figure 4A.3. The statistical differences between particle sizes of different HPH milks have been indicated using letter grouping (a to e) in the figure. The term “apparent particle size” is influenced by refractive index, density and composition of casein micelles and serum. These factors were expected to change with different homogenization pressures, affecting light scattering patterns and particle size estimations. The
The difference in apparent particle size between 0 MPa (218.5 ± 15.5 nm) and 100 MPa (183.3 ± 15.5 nm) milks were not statistically different ($P>0.05$). Further the particle size increased to 403.7 ± 15.5 nm at 200 to 400 MPa and dropped again to 284.8 ± 15.5 nm at 500 MPa homogenization pressure. The apparent particle size of casein micelles in 0 MPa milk was found to be significantly different from 300 and 400 MPa milks ($P<0.02$). The reduction in casein micelle size at 500 MPa compared to 400 MPa milk was significant ($P<0.01$). The turbidity represented as absorbance values of the milk samples is a good indicator of the size of casein micelles as well (Figure 4A.4). The absorbance values reduced up to a pressure of 200 MPa and consistently increased up to 500 MPa. This is similar to the initial disruption and aggregation observed from the apparent particle size data (correlation between particle size and absorbance data for 100 to 500 MPa HPH milks was $r = 0.63$, $P <0.05$).

**STEM.** The data on apparent particle size was also in agreement with the STEM images of casein micelles from homogenized milks (Figure 4A.5). STEM images showed changes in micelle particle size and also changes in uranyl acetate staining patterns. Uranyl acetate associates with polar groups in proteins (Watson, 1958) and it was hypothesized that the different staining patterns were consequences of changes in the density and porosity of the casein micelles. STEM images of casein micelles homogenized at 0 MPa exhibited the typical porous structure associated with native casein micelles (Trejo and Harte, 2010). The open structures of the micelles provided accessibility to uranyl acetate resulting in strong staining. When the milk was homogenized at 100 MPa, the casein micelles partially disrupted, but retained their open structure as observed by the reduction in micelle size and the presence of uranyl acetate staining within the micelle. However, at 200 MPa pressure, the uranyl acetate dye stained only the
outside surface of the casein micelles, creating a stain halo around smaller micelles. It was hypothesized that micelle compaction and loss of porosity explained the inability of uranyl acetate to penetrate the interior of the casein micelles. Similar increase in packing density of proteins was observed in earlier studies (Balny and Masson, 1993, Masson, 1992) on applying high hydrostatic pressure treatment above 200 MPa. Smaller and compact casein micelles aggregated together forming larger micelles at 300 MPa in the present study. The size of casein micelles continued to increase as homogenization pressure reached 400 MPa indicating increased association among compact moieties. This micelle to micelle association could be explained by the complex formation of denatured whey proteins and caseins enabling smaller compact casein moieties to aggregate into bigger micelles. This phenomenon may have been complemented by the utilization of pasteurized milk for HPH which already has denatured whey proteins (Rynne et al., 2004). The increased denaturation of whey proteins at higher homogenization pressures has been shown in the SDS-PAGE gels (Figure 4A.2). The increase in aggregation and size of casein micelles from 100 to 400 MPa milks can therefore be associated with the complex formation with denatured whey proteins. Other mechanisms indicate the association of casein micelles due to lack of steric repulsion owing to the collapse of κ-casein on HPH (Escobar et al., 2011, Roach and Harte, 2008). Another suggested mechanism indicated that hydrostatic pressure causes hydration of hydrophobic amino acids which in turn increase the volume of protein tertiary and quaternary structures (Hummer et al., 1998). However, in the present study the lack of uranyl acetate staining in the interior of the casein micelles on milk homogenized at pressure above 200 MPa indicated an increase in density (or less hydration) of newly formed casein micelles. Further, in agreement with apparent particle size results, STEM images showed that
newly formed aggregates dissociated into smaller micelles at 500 MPa HPH pressure. Apparently the forces acting on the micelles induced by the HPH overcame the forces interacting within the micelles at 500 MPa HPH pressure and thus dissociating it.

All these observations indicated the presence of three different types of casein micelles on the application of HPH pressures up to 500 MPa. They are the highly porous native casein micelles, the compacted casein micelles, and the aggregations of compacted casein micelles. These aggregations have also been referred to as casein micelles in the present study as they also stay dispersed as colloidal moieties in the milk system. The suggested structural changes in casein micelles processed at different HPH pressures are shown in Figure 4A.6. The open porous structure of casein micelles in 0 MPa milk has previously reported in pasteurized milk (Dalgleish, 2011, McMahon and Oommen, 2008, Trejo et al., 2011). The use of HPH pressure of 100 MPa reduces the size of the casein micelles due to partial disruption of the micelles but STEM images suggest that no major changes occur in porosity or density. At 200 MPa homogenization pressure, dissociation and compaction of the casein micelles occur. As the pressure was increased to 300 and 400 MPa, shear induced increase in temperature in the homogenization valve promoted strong hydrophobic interactions resulting in micellar re-association. This increase in micellar size could be driven by both casein-casein interactions and casein-whey protein interactions. The ultra-HPH pressure of 500 MPa induces much higher forces on the casein micelles in the form of shear, cavitation, hydrostatic pressure, impingement, and turbulence compared to lower HPH pressure. These forces overcome the internal forces within the casein micelles and cause re-dispersion of casein micelles to form smaller moieties at 500 MPa pressure.
**pH.** pH values of the different HPH milks were measured. A relatively small but statistically significant increase of ca. 0.13 pH units was observed after milk homogenization at 100 MPa vs. untreated samples (Figure 4A.7). Such small variations could be technologically significant in the case of manufacture of products (e.g., cheese) which are dependent on the ionic balance in the milk system for its product characteristics (Marchesseau et al., 1997). The pH of milk reached a maximum of 6.94 ± 0.01 after homogenization at 100 MPa and then it decreased as homogenization pressure increased from 200 MPa (pH 6.9 ± 0.01) to 500 MPa (pH 6.86 ± 0.01). The opposite was observed by Zamora et al., 2007 who reported a drop in pH to 6.52 when raw milk with 3.5% fat was processed at 100 MPa vs. milk processed at 0 or 300 MPa that showed pH of about 6.72. This variation in the observations of different research groups could be associated with the difference in the type of milks and the pressure systems used including the temperature and other treatment conditions. The pH variation in milk is also associated with a number of physicochemical factors including ionic calcium in the serum phase, exposure of charged and uncharged amino acid residues, and casein dissociation. It is known that high pressure homogenization promotes the migration of calcium phosphate from within the casein micelles to the serum phase with pressures up to 300 MPa (Roach and Harte, 2008, Zamora et al., 2007). Further investigations on the ionic changes occurring in milk are needed to gain better understanding of the precise factors associated to HPH.

**Shelf stability.** The milk samples collected after homogenization exhibited thicker consistency as pressure increased from 0 to 500 MPa. However, all milk samples were shelf stable for at least 14 days at 4°C and no phase separation or settling was observed. Moreover, there was no evidence for spoilage or settling in milk samples processed at 500 MPa even after 2 months of
storage at 4°C, while in all the other samples visible spoilage was evident. The Newtonian viscosity of the skim milks was studied and found to increase when the homogenization pressure was increased from 200 to 500 MPa (Figure 4A.8). The viscosity of 0 MPa (2.2 ± 0.33 mPa.s) and 100 MPa (1.76 ± 0.33 mPa.s) milks are not significantly different (P > 0.05). The milk subjected to higher homogenization pressures of 200, 300 and 400 MPa show consistent increments in viscosity and the 400 (4.13 ± 0.33 mPa.s) and 500 MPa (4.19 ± 0.33 mPa.s) HPH milks showed significantly higher viscosity compared to 0 MPa milk (P < 0.05). There is high correlation of dynamic viscosity and apparent casein micelles size as both have higher values at higher HPH pressure (r = 0.82; P < 0.01). In the present study, the changes induced in particle size are owing to compaction in the casein micelles and its aggregation. These structural changes can be again correlated to increase in viscosity of the milks on HPH. Heat denaturation of whey proteins especially β-Lg and its complex formation has also been reported to increase turbidity and viscosity in milk samples (Anema and Li, 2003). However, the contribution of whey proteins in the structural changes of casein micelles need to be further investigated.

**Studies on milk stability**

**pH stability.** The apparent casein micelle average diameter and turbidity (absorbance of 550 nm) of the milk samples subjected to 0, 400 and 500 MPa homogenization pressures and adjusted to pH 2 to 10 were determined. The apparent particle diameter and absorbance values of samples with pH adjusted below 5 are not shown as these samples precipitated (Figure 4A.9 and 4A.10). At pH 5, milk homogenized at 500 MPa exhibited larger apparent particle size than the milk homogenized at 400 MPa and 0MPa (528 ± 55 nm, 307 ± 17 nm, and 253 ± 13 nm, respectively), but no difference in stability was observed (no sediment formation). The apparent
casein micelle diameter decreased consistently over a pH of 6 to 10 (Figure 4A.9) and all samples turned translucent at pH 10 (Figure 4A.10). The visual appearance of milk homogenized at 500, 400 and 0 MPa and adjusted to pH 7, 8, 9 and 10 is shown in Figure 4A.11. As indicated before, changes in refractive index of the milk serum could explain the difference between apparent casein micelle size measured by light scattering and spectrophotometric observations. The difference between the absorbance values of milk homogenized at 500 MPa and 0 MPa adjusted to pH 5 (Figure 4A.10) was not as evident as for the apparent particle size data (Figure 4A.9). There are variations in the trend for casein micelle size and turbidity measurements for milk subjected to 0 MPa milk compared to milk homogenized at 500 and 400 MPa at pH above 7 as well. Despite of all these variations in trends, a high positive correlation between the absorbance and particle size have been observed for 0 MPa ($r = 0.937, P<0.01$), 400 MPa ($r = 0.971, P<0.01$) and 500 MPa ($r = 0.844, P=0.03$) HPH milks. A lower turbidity of 500 and 400 MPa at pH 8 and above has been observed. The reduction in apparent particle diameter at pH above 8 was consistent with results from earlier studies indicating disruption of hydrogen bonds leading to micellar dissociation at alkaline pH (Liu and Guo, 2008, Vaia et al., 2006, Zhong et al., 2007). At alkaline pH of above 8, intermolecular disulphide linkages are promoted among whey proteins (Patel et al., 2006, Van Camp and Huyghebaert, 1995) reducing their ability to form complexes with caseins. These phenomena could be responsible for the reduction of the aggregation of casein micelles and in turn the absorbance values of milks (0, 400 and 500 MPa HPH milks) adjusted to alkaline pH conditions.
**Ethanol stability.** The percentage ethanol required for the coagulation of milk proteins at pH between 6.1 and 7.1 was studied for HPH milks (Figure 4A.12). The increase in pressure of homogenization lead to the reduction of the percentage ethanol required to coagulate milk. This is evident from the significantly lower values for the percentage ethanol required to coagulate 400 and 500 MPa milk compared to 0 MPa milk ($P<0.05$). Similar reduction in ethanol stability has been reported in milks subjected to hydrostatic pressure (Huppertz et al., 2004). The reduction in ethanol stability at lower pH might be due to the increased solubilization of colloidal calcium phosphate from within the casein micelles. The ethanol draws out the colloidal calcium phosphate within the casein micelles and this counteracts its destabilizing effect on the casein micelles (Horne, 1987, Ye and Harte, 2013). Hence, the solubilization of colloidal calcium phosphate increases the susceptibility to ethanol of casein micelles at lower pH for different HPH milks and thus reduces its stability. The observations from STEM image (Figure 4A.5) indicates that the HPH milk (above pressure 100 MPa) have compact moieties loosely held together to form bigger micelles. HPH has been associated with the complex formation of denatured $\beta$-Lg with caseins and/or the loss of the $\kappa$-casein hairy layer from the surface of the casein micelles (Escobar et al., 2011, Roach and Harte, 2008, Zobrist et al., 2005). This could lead to the exposure of more hydrophobic groups on the surface of the resultant casein micelles, held loosely together making them more accessible to ethanol. A better understanding of the ionic calcium content and reformed structure of the casein micelles can lead to configuration of the specific action of ethanol in an HPH milk system.

**Heat stability.** The heat stability of the milks homogenized at pressures 0 to 500 MPa were studied at 120°C for 3, 6, 12 and 24 min with pH adjusted to 6.3 to 6.7 (with increments of pH
As the homogenization pressure was increased from 0 to 500 MPa, milks exhibited increased susceptibility to form aggregates and gels in response to thermal processing at pH lower than 6.6. The milks homogenized up to 500 MPa were stable at normal milk pH of 6.6 and 6.7 even after 24 min at 120ºC. The heat stability of proteins has been associated with the amount of exposed hydrophobic residues (Considine et al., 2007) and studies using high hydrostatic pressure treatments indicated unmasking of hydrophobic regions in milk proteins (Masson, 1992). However, contrary to our observations using high pressure homogenization, the processing of milk using high hydrostatic pressure up to 600 MPa has been reported to increase the heat stability of milk at pH above 6 (Huppertz et al., 2004).

**Rheology of HPH milk rennet coagulated gel**

The gelling time induced by rennet addition increased with increasing homogenization pressure (Table 4A.3). There was a significant increase in the gelling time when the milk was homogenized at 400 MPa compared to the milk homogenized at 0 to 300 MPa. At 500 MPa homogenization pressure, the milk lost its coagulation capability altogether as indicated by the lack of rennet gelation for 90 min after the addition of rennet. The maximum G’ values decreased from 101.9 ± 4.4 to 0.63± 4.4 Pa, when the homogenization pressure increased from 0 to 500 MPa. The maximum G’ values of milk pressurized at 400 (5.86 ± 4.4 Pa) and 500 MPa (0.63± 4.4 Pa) was significantly lower than the rest of the milks (P<0.01). The 0 MPa milk had the highest maximum G’ (101.9 ± 4.4 Pa) compared to all other milks (P<0.01). Although the reason or the inability of milks processed at 500 MPa to respond to enzymatic coagulation is not known, a number of factors including the stabilizing properties of κ-casein on the casein micelles, and calcium sensitive β-casein concentration in the serum phase, are associated with
rennet coagulation and may explain this phenomenon (Choi et al., 2007, Portnaya et al., 2008). Further studies to understand the concentration of specific casein within the micelles and in the sera is essential to better explain the changes in renneting characteristics with HPH in milk.

**Conclusions**

The study of physicochemical characteristics of milk homogenized up to 500 MPa indicated variations in the particle size of the casein micelles, structure of casein micelles, viscosity, and stability to various pH, heat and concentrations of ethanol. Homogenization up to 500 MPa did not considerably affect the shelf stability of the milk samples over 14 days at 4°C. The reasons for these variations in the apparent size and structure of the casein micelles can be associated with a number of changes in the milk system. The particle size of the casein micelles increased with increase in homogenization pressure from 100 to 400 MPa and then decreased at 500 MPa. Our study also revealed changes in the porosity and density of homogenized casein micelles as evident by the lack of uranyl acetate staining within the casein micelles. The casein micelles existed as porous structures at 0 and 100 MPa pressure, compacted in to a tight structure at 200 MPa and aggregated to increase in size until 400 MPa. At 500 MPa the HPH forces re-dispersed the casein micelles. The exact permutation and combination of different factors involved in ultra-HPH processing responsible for the changes in the casein micelles in milk need further elucidation. However this study presents ultra-HPH as a novel technology to modify the structure, physicochemical and technological properties of casein micelles in pasteurized skim milk.
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Appendix

Figure 4A.1: Temperature of ultra-HPH milk soon after collection and the refrigerant pumped from the water bath for cooling the HPH milk spray. Bars indicate the standard error from two replications.
Figure 4A.2: The SDS-PAGE images of A) milk samples at non-reducing conditions and B) supernatant samples at reducing conditions, subjected to homogenizing pressures of 500, 400, 300, 200, 100 and 0 MPa (from left to right).
Figure 4A.3: Apparent particle size (in nm) of casein micelles in pasteurized skim milk homogenized at different pressures (0 to 500 MPa). Bars indicate the standard error from three replications. Different letters indicate the statistical difference between casein micelle sizes at different HPH pressure ($P<0.05$).
Figure 4A.4: Turbidity of casein micelles in pasteurized skim milk homogenized at different pressures (0 to 500 MPa) adjusted to pH 7. Bars at 0, 400 and 500 MPa pressures indicate the standard error from two replications.
Figure 4A.5: Scanning transmission electron microscopy images of milk homogenized at pressure 0 to 500 MPa
Figure 4A.6: Suggested model of casein micelles in pasteurized skim milks subjected to high pressure homogenization at 0, 100, 200, 300, 400 and 500 MPa. The blue lines indicate the caseins, the red lines whey proteins and the blue dots are colloidal calcium phosphate.
Figure 4A.7: pH of pasteurized skim milk homogenized at pressures 0 to 500 MPa. Bars indicate the standard error from three replications.
Figure 4A.8: Newtonian Viscosity of pasteurized skim milk homogenized at pressure 0 to 500 MPa. Bars indicate the standard error from two replications.
Figure 4A.9: Apparent casein micelles size (in nm) in pasteurized skim milk homogenized at pressures 0 to 500 MPa and adjusted to pH from 5 to 10. Bars indicate the standard error from two replications.
Figure 4A.10: Absorbance values of pasteurized skim milk homogenized at pressure 0 to 500 MPa and adjusted to pH from 5 to 10. Bars indicate the standard error from two replications.
Figure 4A.11: Images of milk samples homogenized at pressure of 0, 400 and 500 MPa adjusted to pH of 7, 8, 9 and 10 (from left to right)
Figure 4A.12: Percentage of ethanol solution required to coagulate the proteins in pasteurized skim milk homogenized at pressures 0 to 500 MPa and adjusted to pH from 6.1 to 7.1.
Table 4A.1: The heat stability of the milk homogenized at pressures 0 to 500 MPa indicated as the condition of the milks after heating to 120°C for different time periods (3, 6, 12 and 24 min) after adjusting their pH in the range if 6.3 to 6.7. The different coagulation conditions denoted as NA for no aggregates, S for small aggregates, B for big aggregates and G for gel. Any variation observed between the replicates indicated as the alternate result indicated after ‘/’.

<table>
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<tr>
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</tr>
</thead>
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<tr>
<td></td>
<td>0 MPa</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>120°C for 3 min</td>
</tr>
<tr>
<td>6.7</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>6.6</td>
<td>NA</td>
</tr>
<tr>
<td>6.5</td>
<td>NA</td>
</tr>
<tr>
<td>6.4</td>
<td>NA</td>
</tr>
<tr>
<td>6.3</td>
<td>B/G</td>
</tr>
<tr>
<td></td>
<td>120°C for 24 min</td>
</tr>
<tr>
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<td>NA</td>
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</tr>
<tr>
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<td>NA</td>
</tr>
<tr>
<td>6.4</td>
<td>NA/S</td>
</tr>
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<td>6.3</td>
<td>G</td>
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Table 4A.2: The coagulation conditions for pasteurized skim milk homogenized at pressures from 0 to 500 MPa after heat treatment at 120°C for different time periods. Any variation observed between the replicates indicated as the alternate result indicated after '/'.

<table>
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<tr>
<th>Pressure</th>
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</tr>
<tr>
<td>100 MPa</td>
<td>pH in 3 min/at 6.4 pH in 24 min</td>
</tr>
<tr>
<td>200 MPa</td>
<td>pH in 12 min/at 6.4 pH in 24 min</td>
</tr>
<tr>
<td>300 MPa</td>
<td>pH in 24 min/at 6.5 pH in 12 min</td>
</tr>
<tr>
<td>400 MPa</td>
<td>pH in 24 min/at 6.4 pH in 24 min</td>
</tr>
<tr>
<td>500 MPa</td>
<td>pH in 24 min/at 6.5 pH in 24 min</td>
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Table 4A.3: Rennet gelling time and maximum G' of pasteurized skim milks homogenized at pressures from 0 to 500 MPa

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>Gel time (min)</th>
<th>Maximum G' (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24.6 (5.8)</td>
<td>101.93 (4.39)</td>
</tr>
<tr>
<td>100</td>
<td>21.0</td>
<td>43.08</td>
</tr>
<tr>
<td>200</td>
<td>24.7</td>
<td>59.77</td>
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<tr>
<td>300</td>
<td>32.5</td>
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<tr>
<td>400</td>
<td>64.9</td>
<td>5.86</td>
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<tr>
<td>500</td>
<td>&gt;90</td>
<td>0.63</td>
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CHAPTER 4B
ACID GELATION OF ULTRA-HIGH PRESSURE HOMOGENIZED MILKS WITH AND WITHOUT POST HEAT TREATMENT
This chapter is a lightly revised version of a paper by the same title to be submitted to the Journal of Dairy Science by M. S. Mohan, L. Liu and F. Harte. The use of “our” in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the sample preparation, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the manuscript writing.

Abstract
High pressure homogenization (HPH) of milk to a pressure of 500 MPa is a novel processing method. We report the rheological properties and whey holding capacity of model yogurts obtained from milk homogenized at pressures from 0 to 500 MPa (HPH milks) and milk that homogenized and then thermally treated at autoclaving conditions of 121°C for 15 min at 103.4 kPa pressure (HPH+Heat milks) on acid gelation using 3% w/v glucono δ-lactone. The apparent casein micelles size (by dynamic light scattering), Newtonian viscosity (flow curve) and SDS-PAGE (reducing and non-reducing conditions) analyses were performed on HPH and HPH+Heat milks. G’ was measured at 22°C for 110 min (1.00 Hz frequency, 0.01% strain). Gelation time was defined as the time when G’ ≥1 Pa and firmness the G’ value after 110 min. WHC was calculated based on the amount of whey expelled when centrifuged at 1,000 × g for 10 min (% weight of whey per volume of milk). The firmness of the HPH gels (G’) increased at 100, 200, 300 and 400 MPa pressures (108 ± 5 Pa) as compared to that at 0 and 500 MPa homogenization pressures (76 ± 3 Pa). Firmness of HPH+Heat gels (39 ± 4 Pa) was lower than that of HPH gels. Firmness of HPH gels increased at higher homogenization pressures, owing to the structural changes occurring in the casein micelles and the increased interaction among micelles and with whey proteins, as evident from the casein micelles size and SDS-PAGE analysis. Gelation time of HPH milks decreased at 300, 400 and 500 MPa (ca. 45 min) compared to that at 0 MPa (ca. 55 min) homogenization pressures. Despite differences in structure, the gelation time required by both HPH and HPH+Heat milk gels (ca. 47 min) was the same. The WHC from HPH milk...
gels decreased slightly at 300 and 400 MPa (ca. 87.8 %w/v) as compared to other HPH milk gels (ca. 93.5 %w/v). For HPH+Heat milk gels homogenized at 300, 400 and 500 MPa pressure (ca. 44.2 %w/v), the WHC was much lower than 0 MPa HPH+Heat gel (ca. 98.3 %w/v). The results indicate that changes occurring within the homogenized milk samples and post homogenization thermal treatments induce changes in the acid gelation properties of milk samples. The HPH pressures up to 500 MPa and in combination with thermal treatment had a significant effect on the physicochemical and acid gelation properties of pasteurized skim milk.

**Introduction**

Dairy products are an important part of everyday diet and the average per capita yogurt consumption has been increased four times over the past three decades (US Census Bureau, 2011). This is owing to the increasing awareness of their nutritional and health benefits (California Farm Bureau Federation, 2013). Yogurt is a fermented dairy product formed by the activity of lactic acid bacteria (e.g., *Lactobacillus bulgaricus, Streptococcus thermophilus*). The main components of the gel structure of yogurt are the major milk proteins (caseins) and milk fat. The rheological properties of yogurt gels have been studied under various conditions of thermal treatment and fat content (Lucey et al., 1998a, Shaker et al., 2000). Milk containing 3.5% fat showed higher firmness compared to low fat or skim milk (Lucey et al., 1998a). This increase in firmness of gels with increase in percent anhydrous fat added was attributed to the contribution of caseins and whey proteins stabilizing the homogenized fat globules in the matrix formation (van Vliet, 1988, van Vliet and Dentener-Kikkert, 1982). Confocal scanning laser microscopy also indicates that fat globules interacted with protein matrix of acid gels (Lucey et al., 1998a). Whey holding capacity (WHC) was decreased 3.5% with reductions in fat content
from 5% to 0% in milk for manufacture of stirred yogurt (Keogh and O'kennedy, 1998). To mitigate loss of WHC and rheological properties, fat replacers (e.g., gums and stabilizers) are added to low fat yogurts (Aziznia et al., 2008, Keogh and O'kennedy, 1998, Lobato-Calleros et al., 2004, Sandoval-Castilla et al., 2004, Tamime et al., 1996). In our study we explore the possibility of utilizing high pressure homogenization to induce structural and functional changes in milk proteins that can potentially alter yogurt gelation properties, including firmness and whey holding capacity.

High pressure treatments utilized until present day include processes where static pressure is applied at longer residence time (High Hydrostatic Pressure, HHP) and dynamic pressure (High Pressure Homogenization, HPH) where a fluid is forces through a small restriction to flow and high pressure occurs at very short residence times. HPH is a recent technology that can be used as a continuous process and where the fluid food (e.g. milk) is simultaneously subjected to several physical phenomena including hydrostatic pressure, shear stress, cavitation, turbulence and high temperature (Datta et al., 2005, Diels and Michiels, 2006, Donsì et al., 2006).

Slow acidification of milk using weak organic acids (e.g., glucono δ-lactone, GDL) is commonly used to model yogurt gelation. Although there are differences in the modes and rates of action of the bacterial and GDL acidification, GDL it is often used as to simulate yogurt gelation owing to its simplicity and reproducibility (Banon and Hardy, 1991, Banon and Hardy, 1992, Harwalkar and Kalab, 1980, Hashizume and Sato, 1988, Johnston et al., 1992). Acid gel formation mainly occurs by proteins aggregation when pH drops to the isoelectric point of the casein proteins. This includes two destabilizing actions including elimination of steric repulsion due to the collapse of hairy κ-casein layer and neutralization of the electrostatic repulsions, which stabilize the micelles
(Roefs, 1986). This causes the formation of tightly fused casein micelle aggregate clusters during acidification and gel formation (Kalab et al., 1983).

Apart from the caseins in milk, whey proteins also tend to contribute to the gel structure especially in their denatured form. Heating milk to above 70°C partially denatures the whey proteins, both α-Lactalbumin (α-La) and β-Lactoglobulin (β-Lg). The degree of denaturation is dependent on the time period and temperature of heating. Denaturation exposes the thiol groups of the whey proteins and promotes disulphide interactions with κ-caseins on the casein micelle surface. The whey protein coating on the casein micelles prevents their extensive aggregation on reducing pH to the isoelectric point (~pH 4.6) of casein. The thin and more uniform gel structure produced has more firmness as there are more interactions between the proteins and higher whey retention owing to its greater ability to uphold whey within the matrix (Hernández and Harte, 2008, Serra et al., 2009).

Whey protein denaturation is also induced on HHP (Dufour et al., 1994, Tanaka et al., 1996, Walsh-O'Grady et al., 2001). β-Lg denatures much faster under pressure than α-La (Harte et al., 2003, Hinrichs et al., 1996, Needs et al., 2000a, Penna and Barbosa-Cánovas, 2007, Walsh-O'Grady et al., 2001). However, the effect of denatured whey proteins in pressure treated milk acid gels is different from that in heat treated milk acid gels (Hernández and Harte, 2008, Lodaite et al., 2009, Serra et al., 2009). The gels produced from HPH of skim milk (0 to 300 MPa) had lower gel firmness (0.33N at 300 MPa pressure) than milk samples heat treated at 90°C for 90sec (0.98N; (Serra et al., 2008)). However a combination of HPH (up to 300 MPa) and thermal treatment (90°C for 5 min) was found to increase the firmness of acid gels from skim milk as compared to application of these treatments alone (Hernández and Harte, 2008). There is a
potential of utilizing such combination treatments of pressure and heat to enhance the gelation and yogurt characteristics from skim milk. Hence in our study we utilized higher homogenization pressures up to 500 MPa and a combination of thermal treatment and high pressure homogenization to process skim milk and study the effect of these treatments on their acid gelation properties on acidifying with GDL.

**Materials and methods**

*Sample preparation*
Store bought pasteurized skim milk was subjected to high pressure homogenization (HPH) using a Flow Hyper jet pump (Flow International Corp., Kent, WA). The milk was homogenized at pressures of 100, 200, 300, 400 and 500 MPa and compared with non-homogenization treatment (0 MPa). These samples were referred to as HPH milks. The outlet temperature after homogenization was maintained below 20°C using a heat exchanger. In another treatment, the milk was subjected to a combination of high pressure homogenization and thermal treatments and the milk so treated was referred to as HPH+Heat milks. These milk samples were prepared by heat treatment subjected to HPH by autoclaving at 121°C for 15 min at 103.4 kPa pressure. The treated samples were stored at refrigeration temperatures until testing.

*SDS-PAGE*
The SDS-PAGE was performed as described elsewhere (Laemmli, 1970) on HPH and HPH+Heat milks. The HPH milk was subjected to ultracentrifugation (Beckman Coulter Optima L-100K ultracentrifuge) in polycarbonate tubes (Beckman Coulter, Inc., Fuller, CA) at 100,000×g for one hour at 20°C to obtain the supernatant. The sediment formed in the HPH+Heat milk samples was also subjected to SDS-PAGE after re-dispersing in distilled water.
to study its composition. The supernatant of HPH and sediment from HPH+Heat milks were also subjected to SDS-PAGE. Apart from these the whey released from HPH and HPH+Heat acid gels were also analyzed by SDS-PAGE. All the samples were subjected to non-reducing conditions (without \(\beta\)-mercaptoethanol and no heating) and reducing condition (with \(\beta\)-mercaptoethanol and heating in a water bath at 95°C for 5 min) for SDS-PAGE. For preparing the loading sample, about 150 \(\mu\)L of sample was mixed with varying amounts of reducing (0.5 M Tris-HCL pH 6.8, glycerol, 10% (w/v) sodium dodecyl sulphate (SDS), \(\beta\)-mercaptoethanol, 0.5% (w/v) bromophenol blue in water) or non-reducing (0.5 M Tris-HCL pH 6.8, glycerol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue in water) loading buffer. The amount of loading buffer in the loading sample and the amount of loading samples were adjusted to the concentration of proteins in the gel to obtain maximum contrast of the bands. The amount of loading buffer and loading sample used in the wells for different samples were: HPH milk – 300 \(\mu\)L and 5 \(\mu\)L, HPH supernatant – 300 \(\mu\)L and 10 \(\mu\)L, HPH+Heat milk and sediment – 350 \(\mu\)L and 8 \(\mu\)L, HPH acid gel whey – 500 \(\mu\)L and 4 \(\mu\)L, and HPH+Heat acid gel whey – 210 \(\mu\)L and 8 \(\mu\)L, respectively. These samples were loaded on an electrophoresis gel (12% Tris-HCl, 12 wells, Ready Gel, BIO-RAD, Hercules, CA) along with a molecular weight standard (Cat. no: 161-0363, BIO-RAD, Hercules, CA). The gel was subjected to an electric current of –200V for 38 min and then stained using Coomassie blue stain. The proteins were identified from previous research published (Jovanovic et al., 2007).

**Viscosity and particle size**
Newtonian viscosity of HPH and HPH+Heat samples was measured using a rheometer (AR-2000; TA instruments, New Castle, DE) equipped with a cone and plate geometry (aluminum, 40
mm diameter, Angle – 0 deg: 59 min: 16 ss, truncation gap – 30 µm) and a temperature
controlled Peltier system set at 25°C. Viscosity was calculated as the slope of a shear strain rate
vs. shear stress flow curve (shear rate ranging from 1 to 50 s⁻¹). Particle size was measured by
diluting the samples 3000 times using imidazole buffer (20 mM imidazole+ 10 mM CaCl₂
adjusted to pH 6.6). The samples were vortexed thoroughly before measuring particle size using
a dynamic light scattering particle size analyzer (165° angle, 1.34 refractive index, 50-µm
pinhole; DelsaNano C, Beckman Coulter Inc., Atlanta, GA). The particle size of the casein
micelles was measured as the hydrodynamic diameter calculated from the average of duplicates
of 70 autocorrelation functions and represented as the mean of two replicates. For both particle
size and viscosity analyses two replicated studies were conducted for HPH and HPH+Heat
samples.

**pH profile**
The pH profile for HPH milk samples were studied by acidifying the samples with 3% GDL and
stirring for 5 min in a conical flask. The acidified samples were then transferred in to three 50
mL centrifuge tubes for every treatment before allowing it to acidify at room temperature. The
pH of separate tubes of the same samples were measured (pH/ATC combination electrode,
Denver Instruments, Bohemia, NY) after 0, 1, 2 and 3.5 h. The pH profile was represented as a
mean of all the HPH samples as the variations between different pressure treatments was found
to be very low (Table 4B.1, maximum coefficient of variation = 2.8%).

**Rheology of HPH and HPH+Heat milk acid gels**
The HPH and HPH+Heat milks were acidified using 3% glucono δ-lactone (GDL) with stirring
for 5 min and performed the rheology testing using a rheometer (AR-2000; TA instruments, New
Castle, DE) equipped with a temperature controlled conical concentric cylinder geometry (15 mm stator diameter, 14 mm rotor diameter, 42 mm height). The samples were subjected to oscillatory testing (storage modulus, G’; 1 Hz frequency; 0.01 % strain) over 110 min at 22°C. A thin layer of low viscosity mineral oil (Cat. No: S55667, Fisher Scientific, Hanover Park, IL) was added to the top of the samples to prevent evaporation during the test. The gelation time was the time when G’ ≥1 Pa and firmness, the G’ value after 110 min. For all rheological analyses three replicated studies were conducted for HPH and HPH+Heat sample gels.

**Whey holding capacity from HPH and HPH+Heat milk acid gels**

The HPH and HPH+Heat milk samples were studied for whey holding capacity. It is calculated from using the following equation:

\[
\text{Whey holding capacity (WHC) = } 100 - \frac{\text{Weight of whey released from each sample} \times 100}{\text{Volume of milk used}}.
\]

The samples were acidified with 3% GDL and stirred for 5 min before allowing it to acidify for 3 h at room temperature. The acid gels thus formed were centrifuged at room temperature at 1000×g for 5 min and then for another 5 min. The upper whey layer was weighed and whey holding capacity was calculated from the above equation to be represented as % w/v. For WHC replicated studies were conducted for HPH (three replicates) and HPH+Heat (two replicates) sample gels. The whey released from HPH and HPH+Heat samples were analyzed using SDS-PAGE under reducing and non-reducing conditions. The amount of loading buffer and loading sample used for different samples were: whey from HPH acid gel- 500 µL and 4 µL, and whey from HPH+Heat acid gel – 500 µL and 10 µL, respectively.
Statistical analysis
Experiments were analyzed as completely randomized design. Statistical analysis was done using the PROC GLM procedure of SAS 9.2 (SAS Institute Inc., Cary, NC). The number of replications analyzed for each property studied has been specified with the description of the studies above. The means and mean separations were tested by ANOVA and Tukey-Kramer multiple comparison method. The means were represented along with the standard error.

Results and discussion

SDS-PAGE
SDS-PAGE of HPH and HPH+Heat milk samples were studied (Figures 4B.1 and 4B.3). SDS-PAGE of the supernatant of HPH samples and sediment of the HPH+Heat samples were done to further understand the contribution of each milk protein to the different phases of the milk system including the serum and colloidal phases (Figure 4B.2 and 4B.4). The presence and absence of disulfide linkages can be demonstrated by performing SDS-PAGE at reducing and non-reducing conditions. Disulfide linkages in milk can form among β-Lg, α-La, κ-casein and within themselves, owing to the presence of sulfur containing cysteine amino acid residues.

SDS-PAGE of HPH milk. When the HPH milk samples were subjected to non-reducing conditions during SDS-PAGE (Figure 4B.1), there was slight reduction in the amount of β-Lg observed with increase in homogenization pressure, which is consistent with the results shown in Chapter 4.1. A number of bands having high molecular weights were also observed for 500 MPa compared to the rest of the HPH milks, presumably due to the formation of complexes and aggregates by whey proteins under non-reducing conditions (Figure 4B.1). Whey protein denaturation and formation of dimers, trimers and aggregates have been reported on application of HHP on β-Lg above 300 MPa pressure (Considine et al., 2007, Considine et al., 2005).
Pressure can reduce the forces stabilizing the \( \beta \)-Lg structure, thus exposing the Cys H121 sulphur containing amino acid to form intermolecular disulphide linkages. \( \kappa \)-Caseins can also exist as dimers and these linkages are also not disrupted in non-reducing conditions (Bouguyon et al., 2006). This promotes its existence as monomers and dimers at lower pressures and as aggregates at higher pressures. The formation of disulphide bonds was demonstrated by intrinsic fluorescence and surface hydrophobicity studies conducted for milk treated at HHP pressures 0 to 400 MPa (Ikeuchi et al., 2001, Tanaka et al., 1996, Valente-Mesquita et al., 1998).

Most of the whey proteins were in the soluble form and were not separated from the supernatant by ultracentrifugation. Hence, they can be observed along with some caseins in the SDS-PAGE gel of the supernatant at reducing conditions (Figure 4B.2). This is an indicator of more self association of \( \beta \)-Lg on HPH rather than complex formation with caseins to become part of the micelles. The formation of covalent bonds, especially \( \beta \)-Lg casein complex, was thought to be the main reason for the increasing particle size and viscosity of milk samples subjected to increasing homogenization pressures up to 500 MPa (in Chapter 4.2). However, the amount of such disulphide linkages is enough to cause significant variations in micelle structure and size as indicated by the SDS-PAGE gels. Also, the amount of caseins in the supernatant decreased with increase in homogenization pressure. This indicates more incorporation of caseins in the casein micelles at higher homogenization pressure without allowing their release, even after ultracentrifugation. There are studies indicating that hydrophobic interactions among caseins are enhanced on high pressure homogenization (Roach and Harte, 2008). Hence, casein micelles have other forces mainly hydrophobic interactions stabilizing its structure, formed by high pressure homogenization. Hydrophobic interactions are favored by pressure induced increase in
temperature (Anema et al., 2005b); leading to the formation of casein micelle chains and clusters (Desobry-Banon et al., 1994, Gaucheron et al., 1997, Huppertz et al., 2004, Merel-Rausch et al., 2007, Roach and Harte, 2008, Shibauchi et al., 1992).

**SDS-PAGE of HPH+Heat milk.** After HPH when the milk samples were subjected to very high thermal treatment at 121°C for 15 min at 103.4 kPa pressure, the milk protein structure undergoes a number of changes. The β-Lg band was not seen in the HPH+Heat milk in the gel under non-reducing conditions (Figure 4B.3). Almost all of the β-Lg present in the HPH+Heat samples associated by disulphide linkages among themselves or with caseins, regardless of the homogenization pressures (bands were observed above the band for the caseins). Complex formation among whey proteins and casein micelles on heating has been reported by many researchers (Haque et al., 1987, Hill, 1989, Jang and Swaisgood, 1990, Mulvihill and Donovan, 1987, Sawyer, 1969). The reorganization of the milk protein system has been reported at 115°C for 15 min. The surface hydrophobicity was lower at this time-temperature combination and enhanced the interactions between caseins and β-Lg, producing a compact and stable structure of the micelles (Yüksel and Erdem, 2005). Sediment was formed at the bottom of the HPH+Heat samples subjected to HPH above 100 MPa. It consisted mainly of caseins and α-La along with traces of β-Lg (Figure 4B.4). The 100 MPa HPH+Heat milk had much lower band intensities compared to the other pressure treatments (Figure 4B.4). This can be associated to an increase in aggregate formation of caseins and whey proteins at homogenization pressures above 100 MPa when further thermally treated.
Physicochemical characteristics of HPH and HPH+Heat milk samples

Casein micelle size. As can be seen from Figure 4B.5, size of the casein micelles were similar when 0 MPa (199 ± 50 nm) and 100 MPa (190 ± 50 nm) homogenization pressures were applied to HPH milks. Thereafter the micelle size increased up to a pressure of 300 MPa (567 ± 50 nm) and then decreased up to 500 MPa pressure (436 ± 50 nm) for HPH milks. The casein micelle size for 300 (P=0.01) and 400 (P=0.03) MPa HPH milks were significantly different from 0 MPa milk. The trend observed in the change in particle size was slightly different from that observed in the previous study (as given in Chapter 4.2) with the same homogenization equipment. This difference was induced by differences in the collection temperatures of the HPH milk samples owing to the order of collection of milk samples between the two studies. The 500 MPa HPH milk was collected initially, followed by the lower pressure milk samples during HPH in the present study, while milk was collected in the reverse order (100 to 500 MPa) in the previous study. The order of collection employed in the present study enabled to maintain a temperature of around 14 to 18°C for all milk samples collected after HPH. The increase in particle size up to 300 MPa homogenization pressure is evidently associated with the increasing β-Lg-casein complex formation and hydrophobic interactions induced by higher pressures of homogenization. The SDS-PAGE results of HPH milk, HPH+Heat milk, HPH milk supernatant and HPH+Heat milk sediment also indicated the presence of disulphide bonds and hydrophobic interactions (Figure 4B.1, 4B.2, 4B.3 and 4B.4). However, the effect of hydrophobic interactions has been suggested as the main reason for increase in particle size in HPH and HHP pressure treatments by other researchers (Anema et al., 2005a, Merel-Rausch et al., 2007, Needs et al., 2000b, Roach and Harte, 2008). The formation of whey protein-casein complexes has been associated with the increase in particle size (30 to 35 nm) of casein micelles on heat treatment.
(Anema and Li, 2003). Hence, the thermal energy produced during HPH is one of the reasons for the increase in micelles size. However the increase (ca. 350 nm) observed was much higher on increasing the HPH pressure to 300 MPa from 0MPa as compared to the slight increase in casein micelle size on heat treatment. Hence, this large difference is mainly due to the hydrophobic interactions acting between the caseins and to a lesser extent due to the whey protein-casein complex formation.

In the case of HPH+Heat samples, all samples showed similar casein micelle size, regardless of the homogenization pressures applied (mean diameter: 253 ± 17 nm; Figure 4B.5). This again indicates the extensive rearrangement of the HPH milk occurring on subjecting to thermal treatment as the heat treatment nullifies the variations observed in casein micelles size on being subjected to further thermal treatment. The heat denatured β-Lg rapidly complexes with itself or caseins and forms larger polymers through multiple disulfide linkages (Galani, 1999, Oldfield et al., 1998). Apart from these interactions, many researchers have also associated non-covalent associations to play an important role in aggregation and protein structure formation (Galani, 1999, Havea et al., 1998, Manderson et al., 1998, McSwiney et al., 1994). The SDS-PAGE gel under reducing and non-reducing conditions can only elucidate the presence or absence of covalent interactions, on subjecting HPH milks to thermal treatment. Hence, more studies are required to study the contribution of noncovalent associations including hydrophobic interactions among caseins and whey proteins when subjected to very intense thermal treatments as utilized in the present study.

**Viscosity.** Viscosity is an indicator of the degree of interaction between micelles. The interactions between caseins and micelles provide more forces to overcome when shear is...
applied to the HPH samples. In the HPH milk samples 0 (2.5 ± 1.2 mPa.s) and 100 MPa (3.1 ± 1.2 mPa.s) milks had similar viscosity (Figure 4B.6). Viscosity increased with homogenization pressure above 100 MPa until 500 MPa. It was similar for HPH samples under 200, 300, 400 and 500 MPa homogenization pressures (mean - 7.5 ± 1.0 mPa.s). HPH milk subjected to 500 MPa homogenization pressure (10.3 ± 1.2 mPa.s) had significantly higher viscosity than 0 MPa milk (2.5 ± 1.2 mPa.s, P=0.03). The increase in viscosity of the HPH milks with increase in homogenization pressures was correlated to the increase in casein micelle size in the samples. The only exception was a slight decrease in particle size after 300 MPa, which was statistically at par with 400 and 500 MPa HPH milks. A similar correlation of particle size and viscosity was observed previously too (Chapter 4.1).

The HPH+Heat milk samples showed no differences in viscosities regardless of the magnitude of homogenization it was subjected to, similar to the trend observed for particle size, which was similar for all HPH+Heat milks. Hence the predominant effect in these samples was due to the thermal treatment which completely changed the nature of the forces interacting with proteins in the HPH milk.

**Rheology of acid gels**

**Firmness.** The storage modulus (G’; Figure 4B.7) of the gels over a period of 110 min indicates the solid like behavior of the gels contributed by the rigidity and number of interactions within the gel matrix (Lucey et al., 1998a, Serra et al., 2009). The G’ value of the acid gel increased for HPH milk from 80.6 ± 5 Pa for 0 MPa milk to 118.2 ± 5 Pa for 100 MPa HPH milk (P<0.01). For HPH pressures of 100 to 400 MPa, the G’ values for HPH milk remained the same (mean: 107.9 ± 5 Pa). There was a significant reduction in the G’ value when pressure increased from
400 MPa (97.2 ± 5 Pa) to 500 MPa (70.7 ± 5 Pa; p=0.02). However, the G’ value for 500 MPa was similar to that of 0 MPa milk (80.6 ± 5 Pa). A previous HPH study indicated no differences in the G’ values of milk acid gels subjected to pressures up to 350 MPa as compared to control milk gels (20 Pa; Hernandez and Harte, 2008). Another HPH study reported an increase in G’ value of HPH milks subjected to pressures up to 300 MPa and related it to the reduction in casein micelle size and therefore an increase in the number of interacting particles (Serra et al., 2007). However, in the present study there was an increase in particle size and an increase in G’ value with increase in homogenization pressure. This variability in trend between different studies indicate that different homogenization equipment, procedures and control of other parameters like temperature playing an important role in determining the characteristics of the acid gels formed by HPH milks.

The initial increase in G’ value observed from 0 MPa and maintenance at similar G’ values until 400 MPa in HPH milk associated with the increase in interactions between the casein micelles by a combination of electrostatic, disulphide linkages and hydrophobic interactions (Mellema, 2000). A study indicating an increase in the casein micelle size above 200 MPa HPH pressure indicated the reason to be an increase in hydrophobic interactions (Roach and Harte, 2008). This reasoning is also supported by numerous HHP studies which indicated an increase in hydrophobic interaction causing an aggregation of casein micelles when pressure is released (Amador-Espejo et al., 2014, Needs et al., 2000a). Hence, based on the changes occurring in the casein micelles size with the application of HPH, the interactions between the micelles on acid gelation change as well.
The thermal treatment of HPH milk at autoclaving conditions (mean= 38.8 ± 4 Pa) led to a reduction in the G’ value of acid gels compared to 0 MPa HPH milk (80.6 ± 5 Pa). Earlier studies have extensively reported that casein micelles form disulphide linkages with whey proteins on thermal treatment enhancing the firmness of the acid gels formed by such milks (Lucey et al., 1998a, Walsh-O'Grady et al., 2001). However, contradictory results were obtained in the present study, which has been attributed to the very severe thermal treatment employed (121°C for 15 min at 103.4 kPa pressure). Oldfield et al. (1998) observed that the method of heating greatly influenced the degree of association of denatured whey proteins with casein micelles and rapid heating by direct steam injection led to formation of big whey protein aggregates. Such aggregates did not associate with micelles as compared to smaller aggregates formed during slow heating. Moreover, thermal treatments are known to disrupt covalent and non-covalent bonds, which could lead to extensive rearrangement of casein micelles on heating (Penna and Barbosa-Cánovas, 2007). In association with this a reduction in gel firmness on subjecting milk to UHT treatment and on heating at 90°C for 30 min before acid gelation has been reported (Labropoulos et al., 1981, Lucey et al., 1997, Parnell-Clunies et al., 1987). Hence, the whey protein denaturation and disruption of various bonds and linkages in the milk hinder the formation of strong gels after HPH+Heat treatment of milks.

**Gelation time.** The gelation time was another rheological parameter that was measured as the time required for the acid gels to be formed at conditions of G’≥ 1 Pa (Figure 4B.8). For 0 MPa milk, the gelation time was highest (55.3 ± 2 min) and then reduced with an increase in homogenization pressure up to 500 MPa (42.5 ± 2 min; P<0.01)). This is a very significant reduction in acid gelation time for the food processing industry. This faster formation of acid gel
matrix for 500 MPa HPH milk is due to the presence of more interactions between the micelles, mainly hydrophobic interactions, which makes it easier for the caseins to associate faster among themselves. Moreover, the viscosity of 500 MPa HPH milk was significantly higher than 0 MPa milk (Figure 4B.6). The gelation time was similar for HPH milks homogenized at pressures 0, 100, and 200 MPa. Another group with similar gelation time was milks subjected to homogenization pressures of 100, 200, 300, 400, and 500 MPa. Higher aggregation rates have been reported earlier for HPH milks subjected to pressures of 100 to 300 MPa. However, the characteristics of the casein micelles were very different compared to the present study. In that study, a reduction in particle size with increase in homogenization pressures was reported (Serra et al., 2008). The reduction in gelation time can be related to the viscosity of HPH milk, which also increases with increase in the HPH pressure. The viscosity is attributed to interacting forces between micelles (Bouchoux et al., 2009), which enhances their rearrangements into acid gels.

In the case of HPH+Heat milks, gelation remained similar for milks homogenized at pressures from 0 to 400 MPa (46.8 ± 1 min). The gelation time significantly decreased for HPH+Heat milk subjected to 500 MPa (39.8 ± 1 min). This trend was very similar to that observed for HPH milks. A similar higher degree of association in 500 MPa HPH+Heat milks, must be the reason for the enhanced rate of gel formation. This indicates that the severe thermal treatment did not completely rearrange/affect the milk protein association in 500 MPa HPH+Heat milk samples compared to other HPH+Heat milks.

**Whey holding capacity of acid gels**

The whey holding capacity (WHC) of milk acid gels represented as % w/v is an indicator of the ability to hold whey within the gel matrix (Figure 4B.9). There were only slight differences in
the WHC from different HPH milk gels with the maximum difference between 0 MPa (97.7 ± 2 %w/v) and 300 MPa (88.3 ± 2 %w/v whey; P=0.04), and 0 MPa and 400 MPa (87.3 ± 2 %w/v whey; P=0.04). Thus HPH slightly reduced the whey holding capacity of acid gels formed, compared to 0 MPa pasteurized skim milk. The reason for this reduction can be attributed to the differences existing between the forces stabilizing the whey within the gel matrix for 0 MPa milk and HPH milk, especially the increase in hydrophobic interactions with application of HPH (Roach and Harte, 2008). The whey released from the different HPH milk gels including 0 MPa milk gel showed very similar protein composition on subjecting to SDS PAGE under reducing conditions (Figure 4B.10A).

The WHC (% w/v) for HPH+Heat milk gels were much lower for 300 MPa (47.8 ± 3 % w/v; P<0.01), 400 MPa (42.3 ± 3 % w/v; P<0.01), and 500 MPa (42.6 ± 3 % w/v; P<0.01) as compared to 0 MPa HPH+Heat milk gel (98.3 ± 3 % w/v). Likewise, the WHC (% w/v) by 0 MPa and 100 MPa (97.6 ± 3 % w/v) HPH+Heat milk gels were very similar. Above 100 MPa, the WHC decreased significantly through 200 MPa (75.4 ± 3 % w/v; P<0.01) until 300 MPa (47.8 ± 3 % w/v; P<0.01) HPH+Heat milk gel. This huge reduction in the WHC was associated with increased inability to retain the whey within the gel matrix owing to changes in the protein-protein interaction. Most previous studies indicate an increase in WHC with an increase in whey protein denaturation by heating of milk (Augustin et al., 1999, Mottar et al., 1989, van Marle, 1998). However increase in the severity of heat treatment beyond a maximum value of denaturation of whey proteins does not improve the WHC of acid gels (Dannenberg and Kessler, 1998, Lucey et al., 1998b). Yet another reason, enhancement of hydrophobic interactions at higher HPH pressures (Roach and Harte, 2008), that induce interactions within whey proteins.
and caseins (Needs et al., 2000b), reducing their interactions with each other. This replacement of one set of interactions with other leads to reduction of differences between the G’ values of different HPH+Heat milks (Figure 4B.7). However, the exact changes occurring in the structure and forces stabilizing the structure, for such samples are not known. Further studies are therefore required to elucidate the mechanism of rearrangement and gelation of the casein micelles in HPH milks subjected to severe thermal treatment conditions as employed in the present study.

**Conclusions**

The high pressure homogenization treatment alone and HPH together with thermal treatment produced changes in a number of physicochemical and technological properties of milk proteins, especially the casein micelles. The increase in particle size, viscosity and firmness of acid gels from ultra-HPH milk samples indicate re-arrangement of the casein micelles. The structure of the micelles is stabilized mainly by hydrophobic interactions and to a lesser extent by disulphide and ionic bonds after ultra-HPH. The high thermal treatment of the milk after ultra-HPH induced extensive rearrangement in the milk protein structures. It changed the interactions of casein micelles among themselves and led to formation of small and large whey protein aggregates and casein complexes. These were also concluded to interact between each other and the interacting forces remain predominantly hydrophobic. These variations in the physicochemical characteristics induced changes in the technological properties of the milk that is acid gelation. This was studies based on the rheological properties of acid gels formed by HPH and HPH+Heat milks. The firmness of the acid gels increased with increase in pressure owing to the hydrophobic interaction and complex formation among milk proteins. A corresponding increase in gelation time and decrease in whey holding capacity was also observed. These attributes also
are related to the forces stabilizing the acid gel structure which is modified with the application of HPH. The application of thermal treatment after HPH caused extensive rearrangement of the casein micelles leading to the formation of casein micelles with similar size and viscosity. This similarity was also reflected in the rheology of all the milk acid gels subjected to different HPH pressures before thermal treatment. Hence, the major factors influencing the properties of the milks subjected to HPH and HPH along with thermal treatment are the forces stabilizing the casein micelles and the casein micelle structure. Further studies are therefore required to elucidate the exact structural modifications and the interactions contributing to it, to better understand the reason for the numerous changes in the properties of milk.
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Appendix

Table 4B.1: pH profile of HPH milks on acidifying with 3% glucono δ-lactone

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pH</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.73</td>
<td>0.03</td>
</tr>
<tr>
<td>60</td>
<td>4.95</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>4.34</td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>3.96</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4B.1: SDS-PAGE gels under non-reducing conditions for HPH milks subjected to homogenizations pressures of 0, 100, 200, 300, 400 and 500 MPa. The order of samples from left to right are 500 to 0MPa
Figure 4B.2: SDS-PAGE gels under reducing conditions for supernatant (obtained from ultracentrifugation) from HPH milks subjected to homogenization pressures of 0, 100, 200, 300, 400 and 500 MPa and. The order of samples from left to right are 500 to 0MPa
Figure 4B.3: SDS-PAGE gels under non-reducing conditions for HPH-Heat milks subjected to homogenizations pressures of 0, 100, 200, 300, 400 and 500 MPa. The order of samples from left to right are molecular weight standard and 0 to 500MPa
Figure 4B.4: SDS-PAGE gels (under reducing conditions) for the sediment of HPH+Heat milks subjected to homogenizations pressures of 0, 100, 200, 300, 400 and 500 MPa. The order of samples from left to right are 500 to 100 MPa, molecular weight standard
Figure 4B.5: Casein micelle size (diameter in nm) in HPH and HPH+Heat milks subjected to homogenization pressures 0, 100, 200, 300, 400 and 500 MPa. Error bars indicate the standard errors. The letters indicate the statistical difference between values at different homogenization pressures for HPH (capital letters) and HPH+Heat (small letters) milks.
Figure 4B.6: Newtonian viscosity (mPa.s) of HPH and HPH+Heat milks subjected to homogenization pressures 0, 100, 200, 300, 400 and 500 MPa. Error bars indicate the standard errors. The letters indicate the statistical difference between values at different homogenization pressures for HPH (capital letters) and HPH+Heat (small letters) milks.
Figure 4B.7: Firmness (storage modulus, G’, Pa) of HPH and HPH+Heat acid gels from milks subjected to homogenization pressures 0, 100, 200, 300, 400 and 500 MPa. Error bars indicate the standard errors. The letters indicate the statistical difference between values at different homogenization pressures for HPH (capital letters) and HPH+Heat (small letters) milks.
Figure 4B.8: Gelation time (min) for the formation of HPH and HPH+Heat gels obtained from milks subjected to homogenization pressures 0, 100, 200, 300, 400 and 500 MPa. Error bars indicate the standard errors. The letters indicate the statistical difference between values at different homogenization pressures for HPH (capital letters) and HPH+Heat (small letters) milks.
Figure 4B.9: Whey holding capacity (WHC. %w/v) from HPH and HPH+Heat gels from milks subjected to homogenization pressures 0, 100, 200, 300, 400 and 500 MPa. Error bars indicate the standard errors. The letters indicate the statistical difference between values at different homogenization pressures for HPH (capital letters) and HPH+Heat (small letters) milks.
Figure 4B.10: SDS-PAGE gels (under reducing and non-reducing conditions) for whey from HPH and HPH+Heat gels from milks subjected to homogenization pressures of 0, 100, 200, 300, 400 and 500 MPa and its supernatant (obtained from ultracentrifugation). The order of samples from left to right are: A-1) 500, 400, 500, 300, 200, 100 and 0 MPa, A-2) 500, 400, 500, 300, 200, 100 and 0 MPa, B-1) 0 to 500 MPa, molecular weight standard and B-2) 0 to 500 MPa, molecular weight standard.
CHAPTER 5
CONCLUSION
In the present research work, we studied the size distribution and binding ability to vitamin A of casein micelles, as well as the effect of high pressure homogenization on the physicochemical and technological properties of casein micelles. These studies provide a better understanding of structure-function relationships of bovine native and modified casein micelles. The size of the casein micelles in bovine milk was found to have a broad range between 10 and 693 nm. This indicates high relative polydispersity with values of 0.39 to 0.47. The utilization of cryo-TEM for studying the size distribution enabled the visualization of casein micelles as well. The casein micelles formed spherical and ovoid shapes with irregular peripheries. The mean diameters ($D_{10}$) of 112 to 134 nm for milks from different cows are comparable to sizes obtained from other techniques including dynamic light scattering. This is especially important because casein micelles are flexible structures, and environmental conditions may lead to under and overestimation of the native casein micelle. The high polydispersity of casein micelles was found to be an inherent characteristic even in milk from individual cows.

The vitamin A palmitate in fortified commercially available pasteurized skim milk was found to be associated with the casein micelles (up to 40% of vitamin A present in milk). Many earlier studies indicated the ability of re-assembled casein micelles to act as nanocarriers or microencapsulates for many hydrophobic probes. In the present study we were able to prove that casein micelles in their unmodified form in pasteurized skim milk associated with vitamin A in fortified in milk. No other milk proteins were found to associate with vitamin A apart from the casein micelles. The association of vitamin A palmitate which is a very small hydrophobic molecule is made possible by the hydrophobic forces within the casein micelles together with the presence of channels and cavities in the micelle structure, as reported by Trejo and coworkers.
It is hypothesized that casein micelles can act as a “sponge” soaking the vitamin A in to its cavities owing to the hydrophobic forces stabilizing the internal structure. The vitamin A palmitate associated with the casein micelles was protected from degradation on UV exposure. Only 6 % of the vitamin A added to milk was degraded after 20 mins of UV exposure. This is much lower than the 59% degradation observed for vitamin A added in apple juice. Thus, the association of vitamin A to casein micelles provides protection against photooxidation and photoisomerization on UV exposure. This protection to probes entrapped within the micelles structure can potentially be used to develop delivery systems. However we still need to understand the nature and strength of the interactions between hydrophobic probes and casein micelles to better utilize their binding properties. Although we assumed the major forces stabilizing the association as hydrophobic forces there might be other forces involved (e.g., van der Waal’s forces, hydrogen bonds, and electrostatic interaction). Moreover it would be essential to know the number and location of specific and non-specific binding sites. High pressure homogenization (HPH) up to 500 MPa was found to alter a number of properties of milk. Most of these changes were found to be associated with the changes in casein micelles size and structure. The size of casein micelles increased with increase in HPH pressure. The new larger micelles were formed by the aggregation of smaller compact micelles. Despite of these changes and the increase in viscosity of milk with increase in HPH pressure, the shelf stability of the milks were not affected. The increase in viscosity was associated with the increase in casein micelle size. This higher viscosity values were comparable to those of full fat milks. A slight reduction in the stability of the milks to ethanol, pH and heat due to HPH was also observed. The structural changes occurring in the casein micelles on HPH were associated with whey proteins.
denaturation producing whey protein-casein complexes. Another factor that can affect interactions between casein micelles is the strengthening of the hydrophobic interactions especially during the short time heat exposure during HPH (Roach et al., 2008).

The technological properties of milk subjected to HPH were also studied. The rennet coagulation of milk was affected by HPH. The gelation time increased with the increase in homogenization pressure from 25 to 65 min for 0 to 400 MPa homogenization pressure. Within the 90 min of viscoelasticity analysis, no gel was formed for milks subjected to 500 MPa HPH. The structural changes occurring in the casein micelles on HPH hinder the activity of rennet and formation of the gel matrix. This indicates that the κ-casein is inaccessible for chymosin activity. This could be due to the rearrangement of caseins in the newly formed micelles after HPH. Another reason that can reduce the activity of chymosin enzyme is the complex formation of whey proteins with κ-casein that can hinder its renneting capabilities based on previous studies. Thus, extensive rearrangement in the casein micelles and reformation of new micelles with very different characteristics occur with increase in homogenization pressure up to 500 MPa.

The acid gelation properties of milk samples were also found to change with the application of HPH and its combination with and without post-thermal treatment. The structure of casein micelles were found to be increasingly stabilized by hydrophobic interactions and to a lesser extent by disulphide and ionic bonds based on the observation from the SDS-PAGE gels. These changes in the forces altered the firmness, gelation time and whey holding capacity of acid gels produced using 3% w/v glucono δ-lactone. The firmness of HPH milk acid gels increased with increase in pressure, which could be attributed to the higher hydrophobic interactions within the casein gel matrix. Entirely different forces stabilized the casein micelles on being subjected to a
combination of HPH and thermal treatment. The technological properties of milk were greatly affected by the structural rearrangement (Scanning transmission electron microscopy images) and changes in the size (dynamic light scattering) of the casein micelles in the HPH milks.

Although techniques like cryo-TEM and cryo-tomography have been utilized to understand the structure of the casein micelles, there is still no suitable technique for studying the extremely flexible internal structure of the casein micelles in its native conditions, especially the order or arrangement of the different casein proteins. Hence, new techniques need to be developed to further enable the understanding of the structure of casein micelles.
Maneesha S. Mohan was born in Kerala, India. Growing up she was greatly interested in science and chose computer science, mathematics, chemistry and physics subjects in high school as electives. Later, she chose to pursue her Bachelor of technology (B. Tech.) degree in the field of dairy science and technology. During her Bachelor’s degree, she was amazed by the immense influence of dairy and dairying in the Indian economy and livelihood. After her B.Tech. degree, she joined as Technical Superintendent (Production) at Products Dairy manufacturing ice creams and flavored milks (Ernakulam Regional Cooperative Milk producers Union, Kerala, India). In order to further understand the science of milk and milk products, she decided to pursue her Master’s in the field of dairy science at South Dakota State University under the guidance of Dr. Sanjeev Anand. For her Master’s research, she worked on the enhancement of Conjugated Linoleic Acid (CLA) in Cheddar cheese. She played a lead role in the development, organization and execution of food safety workshops ‘Applied Introductory Food Safety Lab Course I, II and III’ workshops at SDSU (August, 2009; June, 2010 and July 2011), as a part of USDA Higher education Challenge Grant. During this time period she was granted the 2010 and 2011 International Dairy Foods Association /The Dairy Recognition and Education Foundation (IDFA/DREF) Dairy Industry Graduate Scholarship award.

She approached Dr. Federico Harte after his presentation about the possibilities of studying about the structure and properties of the casein micelles in milk. Owing to her interest in understanding the intricacies of the complex structure of the casein micelles in milk, Dr. Harte accepted her as a PhD student. During the course of her PhD, she won the graduate student oral presentation competition conducted by American Dairy Science Association during the Joint Annual meeting.
at Indianapolis, IN (July 2014). During this period, she was also a part of a team representing UT placed second in the Midwest Regional, Institute of Food Technologists (IFT) Student Association College Bowl competition in West Lafayette, Indiana (April, 2011), in Lexington, Kentucky (March, 2012) in Urbana, Illinois (April, 2013), and in Knoxville, Tennessee (April, 2014). She was a recipient of 2012-2014 Edlong Dairy Flavors scholarship award in association with the Institute of Food Technologists. In the future, she plans to pursue a career in the academia doing research in the field of dairy, and to further continue to enjoy her life to the fullest travelling and in the company of her family and friends.