Effects of pulsed electric field processing and ultrasound processing on the physicochemical and functional properties of proteins in milk

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Zheng Liu

31/8/ 2012
Dedication

Dedicated to my beloved parents, father Liu Zhi Gang, mother Xu Bao Li, sister Liu Sui Zhen, and grandma Lin Yu Chun.

Thank you all for your support, sacrifices and love that give me so much strength to overcome many difficulties and pursue my dreams in my life.

In loving memory of my grandpa Liu Song

“A man travels the world over in search for what he needs, and he returns home to find it.”

“离乡别井，游尽世界，只为寻找生命所需；却在回到家的时候，才发现原来生命中最需要的就在眼前。”
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Publications and presentations

Oral presentations:


Poster presentations:


Publications:
Manuscripts submitted to peer-reviewed journals

Food Science & Emerging Technologies.

**Manuscripts in preparation**


# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>α-Lac</td>
<td>α-lactalbumin</td>
</tr>
<tr>
<td>β-Lg</td>
<td>β-lactoglobulin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CMP</td>
<td>Casein macropeptide</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DTT</td>
<td>α-Dithiotheritol</td>
</tr>
<tr>
<td>$E$</td>
<td>Electronic-strength</td>
</tr>
<tr>
<td>$E_s$</td>
<td>Specific energy</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>$f$</td>
<td>Pulsing frequency</td>
</tr>
<tr>
<td>G’</td>
<td>Storage modulus</td>
</tr>
<tr>
<td>G”</td>
<td>Loss modulus</td>
</tr>
<tr>
<td>GDL</td>
<td>Glucono delta-lactone</td>
</tr>
<tr>
<td>HPP</td>
<td>High pressure processing</td>
</tr>
<tr>
<td>HTST</td>
<td>High-temperature-short-time</td>
</tr>
<tr>
<td>HUS milk</td>
<td>Ultrasonicated high-heat milk</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IMCU</td>
<td>International milk coagulating units</td>
</tr>
<tr>
<td>δ</td>
<td>Loss tangent</td>
</tr>
<tr>
<td>LDS</td>
<td>Lithium dodecyl sulphate</td>
</tr>
<tr>
<td>LTLT</td>
<td>Low-temperature-long-time</td>
</tr>
<tr>
<td>MCP</td>
<td>Micellar calcium phosphate</td>
</tr>
<tr>
<td>MES</td>
<td>Morpholino ethane sulphonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>Morpholino propane sulfonic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>$P$</td>
<td>Power input</td>
</tr>
<tr>
<td>PEF</td>
<td>Pulsed electric field</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>$Q$</td>
<td>Flowrate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>SerP</td>
<td>phosphoseryl residues</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical package for the social science</td>
</tr>
<tr>
<td>$t$</td>
<td>Treatment time</td>
</tr>
<tr>
<td>TS</td>
<td>Total solids</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra-high temperature</td>
</tr>
<tr>
<td>UF</td>
<td>Ultrafiltrate</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WPI</td>
<td>Whey protein isolate</td>
</tr>
<tr>
<td>WPC</td>
<td>Whey protein concentrate</td>
</tr>
<tr>
<td>ζ-potential</td>
<td>Zeta-potential</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Pulse duration</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Electrical conductivity</td>
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## Abbreviations of units

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>hr</td>
<td>hours</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>kg</td>
<td>kilograms</td>
</tr>
<tr>
<td>kHz</td>
<td>kilohertz</td>
</tr>
<tr>
<td>kJ</td>
<td>kilojoules</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>kV</td>
<td>kilovolts</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>mL</td>
<td>millilitres</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>nm</td>
<td>nanometres</td>
</tr>
<tr>
<td>Pa</td>
<td>pascal</td>
</tr>
<tr>
<td>ppm</td>
<td>part per million</td>
</tr>
<tr>
<td>µL</td>
<td>microlitres</td>
</tr>
<tr>
<td>µm</td>
<td>micrometres</td>
</tr>
<tr>
<td>µs</td>
<td>microseconds</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
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Summary

Heat treatment is conventionally used in dairy industry to achieve the microbial safety and desired functional properties of dairy products. However, heat treatment can cause detrimental effects on dairy products such as changes in colour and loss of vitamins. Novel processing technologies including pulsed electric fields (PEF) and ultrasound (US) processing have shown great potential to be the alternatives to heat treatment for dairy processing. In this study, the effects of PEF and US processing on milk protein systems that are altered by pH adjustment, EDTA addition or high-heat treatment are examined.

The results show that PEF processing did not affect the structure of the casein micelles or denature the whey proteins in reconstituted skim milk, despite the pH adjustment (pH 6.7 to 8.0) or the EDTA addition (10 to 30 mM). On the other hand, US processing (particularly at 20 kHz) of reconstituted skim milk can disrupt casein micelles and reform micelle-like particles with smaller size and comparable ζ-potential to native micelles. US processing of high-heat treated (90 °C/10 min) reconstituted skim milk can modify the physicochemical properties of casein micelle particles and the heat-induced protein aggregates. Based on the processing effects on the milk proteins, processed milk samples were selected for functionality tests. The results show that milk ultrasonicated at pH 8.0 and adjusted to pH 6.7 had superior renneting properties compared to untreated milks or milk ultrasonicated at pH 6.7.
The concentrated milk (> 40 % total solids) made from milk ultrasonicated at pH 8.0 and adjusted to pH 6.7 had higher viscosity, compared to that made from untreated milk at pH 6.7. However, the GDL-induced acid gels made from high-heat treated milks that were ultrasonicated had similar gelation properties, compared to those made from milks that were only high-heat treated. The heat stability of recombined concentrated milks made from high-heat treated milks that were ultrasonicated was unchanged, compared to those made from milks that were only high-heat treated.

Therefore, PEF processing can be an alternative for pasteurization of milk with preservation of the important milk proteins, although the processing temperature should be carefully controlled. US processing can be integrated to dairy processing to modify the physicochemical properties of milk proteins and achieve desired functional properties.
Chapter 1

General introduction

1.1 Background

Milk is one of the oldest and most important foods of humans. Bovine milk is the major milk consumed throughout the world. Milk can be converted into a vast range of dairy products with unique flavour, sensory and nutritional properties. Ingredients from milk have been used in manufactured food and non-food products (Spreer, 1998). For example, cheese has attractive characteristics such as unique mouth-feel and shelf-stability. It is also easily digestible and is rich in essential amino acids, minerals and vitamins (Spreer, 1998). Cheese accounts for ~ 30% of the total milk usage and is the most popular dairy product in the world (Spreer, 1998). Many other dairy products such as milk powder, yoghurt and concentrated milk have great economic and nutritional importance.

The manufacture of dairy products is highly dependent on the physical and chemical properties of milk protein, which is a key element in terms of the technological functions of milk. Since heat treatments are applied in manufacturing many dairy products to achieve desired functional and nutritional properties (O'Connell & Fox, 2003), it is extremely important to understand the interaction between heat and milk proteins. This has been a popular research subject in dairy science for many decades.
(Anema & Li, 2003a & b; Dannenberg & Kessler, 1988c; Darling, 1980; Kalab, Emmons, & Sargant, 1976; Kudo, 1980; Larsen, Jenness, & Geddes, 1949; O’Connell & Fox, 2003; Schorsch, Wilkins, Jones, & Norton, 2001; Singh & Creamer, 1992; Singh & Fox, 1985; Smits & Van Brouwershaven, 1980). However, with the increasing demand for quality foods, the detrimental effects of heat on milk, such as changes in colour and flavour or loss of vitamins, are being addressed. To avoid these unwanted effects, novel processing technologies including pulsed electric fields (PEF) and ultrasound (US) have been examined for their use in dairy processing. PEF processing can cause microbial and enzyme inactivation (Barbosa-Cánovas, Góngora-Nieto, Pothakamury, & Swanson, 1999). Ultrasound may be used as an alternative for homogenisation, and has effects on cheese ripening or yoghurt fermentation (Gauri, 2005; Villamiel, Van Hamersveld, & de Jong, 1999). These technologies offer the advantages of low energy consumption and are “environmentally-friendly”. Therefore these novel processing technologies are considered to have potential in the future of dairy processing. While most of the studies investigated the effects of these novel processing technologies on the microbial and enzyme inactivation in milk, very few studies focused on the effects of these processing technologies on milk proteins. Notable exceptions include Floury et al. (2006), Yu et al. (2009 & 2012), Nguyen and Anema (2010), Jambrak et al. (2008 & 2010) and Zisu et al. (2011) who examined the effects of PEF or ultrasound on milk proteins. However, there is still a lack of systematic studies on the effects of PEF and ultrasound on milk in well characterized processing systems. In order to integrate
these processing technologies into the dairy industry, the interaction between these processing technologies and the physical, chemical and functional properties of milk proteins needs to be studied and understood to a greater extent.

1.2 Hypotheses:

1) That PEF and ultrasound processing can modify the casein micelle structure and whey proteins in reconstituted skim milk.

2) That PEF- or ultrasound-modified milk proteins have novel functionality.

1.3 Objectives

This study was carried out to investigate the effects of pulsed electric fields and ultrasound processing technologies on the major milk proteins (caseins and whey proteins) that govern the technological properties of milk. The main objectives of this study are listed below:

1) To determine whether two novel processing technologies (PEF and US) disrupt casein micelles or denature whey proteins in reconstituted skim milk
   a) as a function of pH (6.7 – 8.0)
   b) with an added chelating agent (EDTA) at constant pH 6.7 (the native pH of milk).

2) To investigate the effects of ultrasound processing on casein micelles and whey proteins in high-heat treated reconstituted skim milk as a function of pH (6.3 –
3) To investigate the effects of PEF and ultrasound processing on selected functionalities of milk proteins including renneting properties, viscosity, acid-induced gelation, and heat stability.

1.4 Overview of thesis

A literature review is given in Chapter 2 which describes the state of scientific research in the area of milk proteins, and the effects of processing (conventional heat treatments, pulsed electric fields and ultrasound processes) on milk proteins and on the functionality of the milk after these processing treatments. The pulsed electric field processing systems, ultrasound processing units, the key processing parameters, and different analytical techniques that were used in this study are introduced in Chapter 3. In Chapters 4, 5 and 6, the pulsed electric fields or ultrasound processing effects on protein systems in milks that were altered by pH, EDTA or heat are discussed. In Chapter 7, selected milks that were modified by the processing treatments, were used for the studies on the effects of processing on rennet-induced gelation, viscosity during milk concentration process, acid-induced gelation, and heat stability of recombined concentrated milk. Conclusions are presented in Chapter 8 to summarize the findings and outline the important implications of this study.
2.1 Milk

Milk is a complex fluid secreted by mammalian species to supply all the required nutrients for the newborn. It contains water, proteins, carbohydrate in the form of lactose, fat, vitamins and minerals (Fox, 2003). The gross composition of bovine milk is given in Table 2.1.

Table 2.1 Approximate composition of milk. Adapted from Walstra and Jenness (1984a)

<table>
<thead>
<tr>
<th>Component</th>
<th>Average content percentage (w/w)</th>
<th>Range percentage (w/w)</th>
<th>Average percentage of dry matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>87.3</td>
<td>85.5-88.7</td>
<td>N/A</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.6</td>
<td>3.8-5.3</td>
<td>36</td>
</tr>
<tr>
<td>Fat</td>
<td>3.9</td>
<td>2.4-5.5</td>
<td>31</td>
</tr>
<tr>
<td>Protein (casein)</td>
<td>3.25 (2.6)</td>
<td>2.3-4.4 (1.7-3.5)</td>
<td>26 (20)</td>
</tr>
<tr>
<td>Mineral</td>
<td>0.65</td>
<td>0.53-0.80</td>
<td>5.1</td>
</tr>
<tr>
<td>Organic acids</td>
<td>0.18</td>
<td>0.13-0.22</td>
<td>1.4</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>0.14</td>
<td>N/A</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The physicochemical properties of the major milk proteins (caseins and whey proteins) determine the technological properties of milk during the manufacture of many dairy products. Lactose, which makes up about 36% of dry matter, is the principal carbon...
source in milk. As a reducing sugar, lactose also interacts with amino compounds upon heating (Maillard reaction) which is responsible for the colour and flavour in condensed milk (Walstra & Jenness, 1984a). Some important milk salts such as calcium phosphate and citrate, largely contribute to the integrity of the casein micelle which is the most important milk protein complex (Holt, 1997). The concentration of vitamins is very low but they are important in terms of the nutritional properties of milk.

2.2 Milk proteins

There is 30 - 35 g protein/L in bovine milk. Milk proteins are categorized into two groups: caseins and whey proteins. Caseins can be further classed into αs1-caseins, αs2-caseins, β-caseins and κ-caseins. Whey proteins include β-lactoglobulin (β-Lg), α-lactalbumin (α-Lac), bovine serum albumin (BSA), immunoglobulin and proteose peptone (Swaisgood, 1982). The concentrations and some physical and chemical features of milk proteins are listed in Table 2.2.
Table 2.2 Concentration and chemical characteristics of milk proteins. Adapted from Fox (2003), Dalgleish (1992) and Eigel et al. (1984).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amount (g/L milk)</th>
<th>Molecular weight (Da)</th>
<th>Phosphoseryl residues</th>
<th>Sulphhydryl residues</th>
<th>Hydrophobicity (cal/residue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caseins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αs1-casein</td>
<td>10</td>
<td>23,612</td>
<td>8-9</td>
<td>0</td>
<td>1172</td>
</tr>
<tr>
<td>αs2-casein</td>
<td>2.6</td>
<td>25,228</td>
<td>10-13</td>
<td>2</td>
<td>1111</td>
</tr>
<tr>
<td>β-casein</td>
<td>9.3</td>
<td>23,980</td>
<td>5</td>
<td>0</td>
<td>1334</td>
</tr>
<tr>
<td>κ-casein</td>
<td>3.3</td>
<td>19,005</td>
<td>1-3</td>
<td>2</td>
<td>1100</td>
</tr>
<tr>
<td>Whey proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Lg</td>
<td>3.2</td>
<td>18,362</td>
<td>0</td>
<td>5</td>
<td>1019</td>
</tr>
<tr>
<td>α-Lac</td>
<td>1.2</td>
<td>14,174</td>
<td>0</td>
<td>8</td>
<td>1019</td>
</tr>
<tr>
<td>BSA</td>
<td>0.4</td>
<td>~ 66,000</td>
<td>0</td>
<td>35</td>
<td>995</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>0.7</td>
<td>≥ 145,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>0.8</td>
<td>4,000-40,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

2.2.1 Caseins

Caseins represent about 80% of the total milk proteins and they are precipitated at ~ pH 4.6 (Spreer, 1998). The molar ratio of αs1-caseins, αs2-caseins, β-caseins and κ-caseins is 11:3:10:4 (Walstra, Wouters, & Geurts, 2006).

Caseins are all phosphorylated to varying extents as a result of post-translation modifications. αs1-Casein, αs2-casein, β-casein and κ-casein contain 8 to 9, 10 to 13, 5 and 1 to 3 phosphoseryl residues respectively (Dalgleish, 1989). The phosphoseryl residues give the caseins (αs1, αs2 and β) the capacity to bind metal ions, particularly calcium. Dickson and Perkins (1971) have shown that the metal ion binding capacity of the individual caseins increases with the number of phosphoseryl residues. Ono et al. (1976) showed that the infrared absorption band at 980 cm⁻¹, which corresponds to
–PO$_3^{2-}$ in $\alpha_{s1}$-casein, shifts after complexation with Ca. When sufficient Ca is present, $\alpha_{s1}$-, $\alpha_{s2}$-, and $\beta$-caseins can be precipitated and these three caseins are thus categorized as calcium-sensitive caseins. However, $\kappa$-casein which contains 1 to 3 phosphoseryl residue is calcium-insensitive (Horne, 2006).

Their hydrophobicity and the presence of electrical charges are key chemical properties of caseins. The $\alpha_{s1}$-casein molecule has an amphipathic structure composed of a hydrophobic domain and a polar domain. The $\alpha_{s1}$-caseins exhibit high self-association properties, mostly due to the hydrophobic domains in the polypeptide chain. The degree of the association is dependent on the pH and ionic strength of the solution and has been attributed to the anionic clusters in the highly charged polar domains (Swaisgood, 1982). $\alpha_{s2}$-Casein is less hydrophobic than $\alpha_{s1}$-casein. The $\alpha_{s2}$-casein molecule possesses a large net charge (+9.5) in the relatively hydrophobic domain and a large net charge (−21) in the polar domain at pH 6.6. Due to these high net charges in different domains, the associative properties of $\alpha_{s2}$-caseins are largely dependent on the ionic strength (Snoeren, Vanmarkwijk, & Vanmontfort, 1980). $\beta$-Casein is the most hydrophobic casein and its hydrophobic domains have no net charge. Thus $\beta$-caseins form micellar aggregates in solution (Swaisgood, 2003). $\kappa$-Casein is amphipathic and has a distinct separation of the hydrophobic and polar domains. The self-association of $\kappa$-caseins results in a structure similar to that of a soap micelle. The interaction of $\kappa$-caseins with themselves or other caseins is less dominated by hydrophobic interaction, and H-bonding and ion-pair interactions are
also involved (Swaisgood, 1982). A key role of κ-casein is to stabilise the casein micelles (Hill & Wake, 1969).

**Casein micelles**

In normal milk at natural pH ~ 6.7, the phosphorylated caseins interact with each other and also with micellar calcium phosphate (MCP) which is a complex of milk salts ([Section 2.2.3](#)), to form casein micelles. The size of the casein micelle ranges from 15 to 600 nm in diameter, with a mean diameter ~ 200 nm (Rollema, 1992). It is generally accepted that the amphipathic κ-caseins are mostly located on the surface of the casein micelles to terminate the extension of the hydrophobic micelle core and thus limit the size of the micelles. Studies have shown an inverse relationship between the κ-casein content and casein micelle size in milk (Dalgleish, Horne, & Law, 1989; Donnelly, McNeill, Buchheim, & McGann, 1984). The calcium insensitivity of κ-caseins prevents the precipitation of the calcium-sensitive caseins with the calcium in milk. Furthermore, the hydrophilic negatively charged macropeptide region of the κ-caseins diffuses into the solvent and provides stability to the micelle system through electrostatic and steric interactions between the charged micelle particles (Holt & Horne, 1996; Walstra, 1990). The surface charge of the casein micelles is often estimated by measuring the \( \zeta \)-potential of the particles, which can be derived from the electrophoretic mobility of the particles. Various casein micelle structural models have been proposed (Holt, 1992; Payens, 1966; Rose, 1969; Slattery, 1973; Walstra, 1990 & 1999) and the structure of casein micelle still remains controversial. However,
it is generally agreed that hydrophobic interactions, MCP bridging and the micelle surface properties given by the κ-caseins are the key forces responsible for the stability of the casein micelles (De Kruif & Holt, 2003). A structural model of the casein micelle is shown in Figure 2.1.

![Figure 2.1 Structural model of the casein micelle, Holt (1992).](image)

**2.2.2 Whey proteins**

Whey is the liquid remaining after caseins are precipitated from milk. Whey contains proteins, lactose, inorganic salts, vitamins and several trace constituents. The major whey proteins are β-Lg and α-Lac, which represent about 50% and 20% of the total whey proteins respectively (Fox, 2003). β-Lg has a molecular mass of 18 kDa.
Important features of the primary structure of β-Lg are one free thiol group and two disulphide bonds associated with the cysteine residues. After heat denaturation, the β-Lg associates with κ-casein through intermolecular disulphide bonding. α-Lac is a small molecule with a molecular mass of 14 kDa. It has 4 disulphide bonds but no free thiol group, and has Ca binding ability (Fox, 2003).

### 2.3 Milk salts

Milk salts contain calcium, magnesium, sodium, potassium, chloride, phosphate and citrate. The milk salts that can diffuse through a membrane with 10 to 15 kDa MW cut-off are termed diffusible salts. The ones that stay in the retentate are considered to be casein-associated and are called colloidal salts. The typical concentrations of salts and their partitioning behaviours are shown in Table 2.3 (Holt, 1997).

The milk salts (particularly calcium and phosphate) that interact strongly with caseins are commonly referred as micellar calcium phosphate (MCP), due to their key role in stabilizing the structure of casein micelle. MCP, which contains magnesium and citrate as well as calcium and phosphate, can be viewed as a hydrated nano-cluster with a diameter 2.5 nm, surrounded by phosphorylated caseins (Holt, 1997). Thus caseins are also considered as part of the milk salt system.

The salt equilibrium between the micellar (colloidal) and serum (diffusible) phase is closely related to the physicochemical properties of casein micelles. There are
different theoretical models to calculate the salt equilibrium (Holt, 2004; Holt, Dalgleish, & Jenness, 1981; Lyster, 1981; Mekmene, Le Graët, & Gaucheron, 2009; Wood, Reid, & Elvin, 1981). However, there are still no satisfactory models due to the difficulty of taking the complex interaction between salts and caseins into account.

Table 2.3 Concentration (mole/L) of milk salts in bovine milk, adapted from Holt (1997).

<table>
<thead>
<tr>
<th>Component</th>
<th>Total</th>
<th>Diffusible</th>
<th>Colloidal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>30.3</td>
<td>10</td>
<td>20.3</td>
</tr>
<tr>
<td>Mg</td>
<td>5.2</td>
<td>3.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Na</td>
<td>22.8</td>
<td>21.8</td>
<td>1.0</td>
</tr>
<tr>
<td>K</td>
<td>39.7</td>
<td>37.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Pi</td>
<td>21.4</td>
<td>11.9</td>
<td>9.5</td>
</tr>
<tr>
<td>Citrate</td>
<td>9.5</td>
<td>9.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Cl</td>
<td>31.5</td>
<td>31.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

2.4 Protein-mineral equilibria in milk

The partitioning of the micellar and serum phases by ultracentrifugation of milk results in a top layer (fat), a supernatant layer (serum phase), a sedimented pellet (micellar phase), and an opalescent layer as shown in Figure 2.2.
Figure 2.2 Partitioning of skim milk by ultracentrifugation at 55,000 × g for 60 min, 25 °C (The ultracentrifugation conditions are adopted from Hemar et al., 2011).

The equilibrium of milk salts, particularly calcium and phosphate, between the micellar and serum phase is important due to the key role of MCP in maintaining the integrity of the casein micelles (Holt, 1992). Different stresses such as heat, pH or additives are commonly applied to milk to study the milk salt equilibrium. Upon heating of milk to between 90 and 100 °C, the phosphate precipitates as calcium phosphate as shown in reaction 2.1. The insoluble calcium phosphate associates with casein micelles, although this process is reversible at room temperature (Figure 2.3).

3 Ca^{2+} + 2 HPO_4^{2-} \rightarrow Ca_3(PO_4)_2 + 2 H^+ \quad 2.1

The effects of heat on the Ca content in the serum phase (soluble Ca) are shown in Figure 2.3. The liberation of H^+ in reaction 2.1 decreases the pH of the milk (Walstra & Jenness, 1984b). When heating at higher temperature (> 100 °C), the pH of the
milk decreases further due to the hydrolysation of phosphoric acid from caseins and the formation of organic acids from lactose (Walstra & Jenness, 1984b).

Figure 2.3 The amount of Ca in the serum phase in percentage of original amount. (A) Effect of heating at various temperature for various time. (B) Effect of holding time on heated milk (78 °C/30 min) at 20 °C. Adapted from Walstra and Jenness (1984b).

Acidification of milk (by addition of acid, bacterial fermentation or heating) dissolves the MCP and dissociates the Ca from the casein micelles (Holt, 1997; Walstra & Jenness, 1984b). The ionic strength and Ca activity are therefore increased. The effects of acidification on the P, Ca, Mg and citrate in the serum phase of milk are shown in Figure 2.4.
The addition of Ca-chelating agent EDTA in milk decreases the calcium activity in the serum phase, which leads to the dissolution of MCP (Holt, 1992; Holt, Davies, & Law, 1986). With the addition of 30 mM EDTA, 80 % of the total caseins became non-sedimented after ultracentrifugation (Johnston & Murphy, 1992). Ward et al. (1997) and Chandrapala et al. (2010) observed an increase of protein content in the serum phase after the addition of 10 – 20 mM EDTA in milk. Rollema and Brinkhuis (1989) also observed the increase of casein content in the serum phase with EDTA addition by using $^1$H NMR spectroscopy.
2.5 Milk protein functionality

2.5.1 Rennet-induced gelation

The principle of cheese-making is using rennet to coagulate milk into a cheese curd. Rennet is an extract from calf stomach and contains a clotting enzyme, chymosin. The addition of chymosin to milk initiates the primary phase of the clotting reaction by a proteolytic reaction (Hyslop, 2003):

\[ \kappa\text{-casein} + \text{rennet} \rightarrow \text{para-}\kappa\text{-casein} + \text{casein macropeptide (CMP)} \]

Chymosin cleaves the \( \kappa \)-casein on the micelle surface into para-\( \kappa \)-casein and CMP. Para-\( \kappa \)-casein is very hydrophobic. Thus extensive cleavage of \( \kappa \)-caseins will result in the aggregation of casein micelles which is considered the secondary phase of the clotting reaction by rennet. Eventually the casein micelles will form a gel network which is the cheese curd (Hyslop, 2003). A schematic diagram of the attack of chymosin on casein micelles is shown in Figure 2.5.
Figure 2.5 Schematic diagram of the attack of chymosin on casein micelles. (a) Adding chymosin into $\kappa$-casein coated micelles. (b) Chymosin hydrolyses $\kappa$-caseins but sufficient $\kappa$-caseins remain to prevent aggregation. (c) Most of the $\kappa$-caseins are hydrolysed and the micelles start aggregating. Adapted from Dalgleish (1992).

The production of the CMP can be quantified using different methods such as far-UV spectroscopy (Castle & Wheelock, 1972), HPLC (Hooydonk & Olieman, 1982) or ELISA (Picard, Plard, Rongdaux-Gacia, & Collin, 1994) and hence the rennet activity can be measured. The renneted micelles lost half of their negative charges and the surface layer collapses leading to micelle aggregation (Dalgleish, 1992). Rennet-induced gelation properties such as gel firmness, gelation time and gelation rate are commonly studied using rheological methods (Walstra & Jenness, 1984c). Various factors can affect the gelation properties. Choi, Horne and Lucey (2007) showed that rennet-induced gels made from milk at pH 6.4 had higher maximum firmness and more interconnected networks compared to that made from milk at pH 6.7. They also showed that rennet-induced gels made from milk with added EDTA (2 – 6 mM) had a more finely dispersed but weaker and more flexible protein particle
network, compared to that made from milk with no EDTA addition. In addition, the
gelation temperature and milk solids content also have different effects on the
rennet-induced gelation behaviours (Bansal, Fox, & McSweeney, 2007; Mishra,
Govindasamy-Lucey, & Lucey, 2005).

2.5.2 Acid-induced gelation

Yoghurt is made from the fermentation of lactose to lactic acid in milk by bacterial
culture. The decrease of the milk pH to the isoelectric point (~ pH 4.6) of casein
micelles leads to the aggregation of micelle particles due to the loss of balance
between intermolecular attraction and repulsion. In addition, pH reduction also
dissolves the MCP (Figure 2.4) which leads to the liberation of micellar caseins and
the increase of the calcium ion activity in milk. As the proteins lose their charge and
electrostatic repulsion is decreased, the casein particles form an acid-induced gel due
to hydrophobic interactions (Lucey & Singh, 2003). Although the pH-modified casein
particles form the acid-induced gel network, the incorporation of whey proteins into
the network is crucial for improving gelation properties. Heat treatment > 70 °C
causes whey protein denaturation and formed aggregates. These heat-induced whey
protein aggregates have significant effects on the acid-induced gelation which will be
discussed further in Section 2.7.1 (Lucey & Singh, 1997). The rheological properties
of yoghurt gels made from unheated and heated milk are shown in Figure 2.6. The
considerably higher storage modulus (G’) in gel made from heated milk indicates the
significantly higher gel strength, compared to the gel made from unheated milk.

Figure 2.6 Effect of heat treatment on the rheological properties of yoghurt gel made at 40 °C from unheated milk (circles) or milk heated at 82 °C for 30 min (triangles). Solid circles or triangles plot the storage modulus and open ones plot the loss tangent. Open squares correspond to pH measurement. Adapted from Lucey (2002).

2.5.3 Heat stability

Heat processing of milk is commonly used in the dairy industry. Different traditional heating regimes are shown in Table 2.4. Normally, milk is very heat stable due to the flexible structure of casein micelles. However, heat-induced coagulation in milk or concentrated milk can happen during sterilization under certain conditions (O’Connell & Fox, 2003). A commonly used method to measure heat stability is the “subjective heat stability assay”: Milk sample is sealed in a glass tube and place in an oil bath
usually at 140 °C for milk and 120 °C for concentrated milk. The time required for the onset of visual coagulation is measured (O'Connell & Fox, 2003).

**Table 2.4 Common heat treatments applied in dairy industry. Adapted from O’Connell and Fox (2003).**

<table>
<thead>
<tr>
<th>Heat regime</th>
<th>Conditions</th>
<th>Objectives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermisation</td>
<td>65 °C × 15 min</td>
<td>Killing of spoilage microbes</td>
</tr>
<tr>
<td>Pasteurization</td>
<td>63 °C ×30 min</td>
<td>Killing of pathogenic microbes</td>
</tr>
<tr>
<td>LT LT T</td>
<td>72 °C × 15 sec</td>
<td></td>
</tr>
<tr>
<td>HTST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forewarming</td>
<td>90 °C ×2-10 min</td>
<td>Preparatory step for sterilization</td>
</tr>
<tr>
<td></td>
<td>120 °C ×20 sec</td>
<td></td>
</tr>
<tr>
<td>Sterilization</td>
<td>130-140 °C ×3-5 sec</td>
<td>Sterilization</td>
</tr>
<tr>
<td>UHT</td>
<td>65 °C × 15 min</td>
<td></td>
</tr>
<tr>
<td>In-container</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production of specific products</td>
<td>85-90 °C ×5-15 min</td>
<td>Yoghurts and protein coprecipitates</td>
</tr>
</tbody>
</table>

A classic heat coagulation time-pH profile is shown in Figure 2.7. Normally there are two types of time-pH profile due to the genetic variation: Type A milk and Type B milk as shown in Figure 2.7. Type A milk exhibits a maximum and a minimum heat stability at pH ~ 6.7 and pH ~ 6.9 respectively, whereas the heat stability of Type B milk steadily increases as a function of pH. The heat stability of the concentrated milk is much lower. It shows a maximum at pH ~ 6.4 to 6.5 and decreases at both ends of the pH range.
During heat processing of milk, many of the changes that happen can directly or indirectly cause the coagulation. Some of the changes and the possible effects on heat stability are listed in Table 2.5. These changes are closely related to the possible mechanisms of heat-induced coagulation.

**Table 2.5 Changes in milk during heat treatment. Adapted from Singh (2004).**

<table>
<thead>
<tr>
<th>Changes that possibly promote instability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decrease in pH</td>
</tr>
<tr>
<td>Deposition of calcium phosphate onto micelles</td>
</tr>
<tr>
<td>Association of whey proteins with casein micelles</td>
</tr>
<tr>
<td>Dephosphorylation of casein</td>
</tr>
<tr>
<td>Dissociation and hydrolysis of caseins, in particular κ-casein</td>
</tr>
<tr>
<td>Reduction in zeta potential and hydration</td>
</tr>
<tr>
<td>Covalent bond formation</td>
</tr>
</tbody>
</table>
2.6 Process stress-induced changes to milk proteins

Traditionally, heat processing of milk is commonly used to ensure microbial safety and obtain desired functional properties. As mentioned in Section 2.5, the effects of heat and/or pH on milk proteins have been thoroughly investigated and these changes also have effects on milk functionality (Dalgleish & Law, 1988, 1989; Lucey, Singh, H., 2003; Odagiri & Nickerson, 1965; Vandijk, 1992).

In recent years, several new processing technologies have emerged (Wan et al., 2005). These show great potential to impart novel functional properties to milk proteins. These processing technologies include high pressure processing (HPP), pulsed electric fields (PEF) processing and ultrasound (US) processing. The effects of HPP on milk proteins have been relatively systematically studied, covering the physicochemical properties of casein micelles, whey proteins and milk salt systems, and the rennet- and acid-induced gelation behaviours and cheese- and yoghurt-making (Huppertz, Kelly, & Fox, 2002). HPP is not covered further in this review. Investigations of the effects of PEF processing on dairy products have mostly focussed on the antimicrobial effects. The existing knowledge of the US processing effects on the physicochemical and functional properties of milk proteins is very poor. However, some recent studies have showed that PEF and US processing can alter the structure of milk proteins and lead to novel functionalities (Floury et al., 2006; Jambrak et al., 2011; Madadlou, Emam-Djomeh, Mousavi, Mohamadifar, & Ehsani, 2010; Madadlou, Mousavi, Emam-Djomeh, Ehsani, & Sheehan, 2009a; Villamiel & de Jong, 2000; Xiang, Ngadi,
Ochoa-Martinez, & Simpson, 2011; Yu, Ngadi, & Raghavan, 2012). In this section, the effects of pH and heat on milk proteins will be reviewed, followed by a discussion of reported PEF and US processing effects on milk proteins.

2.6.1 pH effects

The natural pH of milk is ~ 6.7. As mentioned in Section 2.4 (Figure 2.4), reducing the pH causes the dissolution of MCP and, consequently, the dissociation of micellar caseins (Dalgleish & Law, 1988). In addition, as discussed in Section 2.2.1, acidification decreases the charge of the caseins and hence facilitates the hydrophobic interaction between caseins. The hydrophobic interaction plays an important role in stabilizing the micelle structure after acid-induced loss of MCP (Rollema, 1992). On the other hand, high alkaline pH can lead to extensive reduction of milk turbidity, which strongly indicates the disruption of casein micelles (Odagiri & Nickerson, 1965; Vaia, Smiddy, Kelly, & Huppertz, 2006; Vandijk, 1992). Huppertz et al. (2007) completely disrupted casein micelles at pH 10.0. When re-adjusting the pH back to natural milk pH (pH 6.6) after the disruption, they observed smaller re-formed casein micelles with similar properties to the native casein micelles. Vaia et al. (2006) suggested that alkaline pH improved the “solvent quality” causing the dissociation of casein micelles. In the pH range of 5.5 to 12.0, Liu and Guo (2008) reported that the casein micelles had looser structure and larger size at higher pH.
2.6.2 Heat effects

2.6.2.1 Heat effects on casein micelles

The effects of heat on casein micelles are dependent on the pH of the milk prior to heat treatment and on the time-temperature profile (Singh & Creamer, 1992). Aoki et al. (1975) have showed that heat treatment (135 °C/15 sec) dissociated 49 % of the κ-caseins, 25 % of the β-caseins and 22 % of the α-caseins. The heat-induced dissociation of micellar caseins was attributed to the removal of Ca in MCP and the disruption of interactions between casein components (Aoki, Suzuki, & Imamura, 1974). Kudo (1980) also showed that high heat treatment (140 °C for 5, 10 or 20 min) of milk at pH range between 6.81 and 7.24 caused an increase of protein content in the serum phase after ultracentrifugation, with the increase being larger at higher pH. Part of this increase of proteins in the serum was due to the dissociation of caseins from casein micelles. At pH < 6.81, heating decreased protein content in the serum because the whey proteins substantially coated the casein micelles and co-sedimented with the casein micelles on ultracentrifugation. Anema and Klostermeyer (1997a) observed that within the pH range 6.3 to 7.1, heat-induced κ-casein dissociation increased with the increase in pH prior to heating. They also showed that within the temperature range of 80 °C to 120 °C, higher heating temperatures caused higher κ-casein dissociation at a given initial pH. At pH > 6.7, high-heat treatment (120 °C or 140 °C) of milk caused the absolute value of the ζ-potential (surface charge) of micelle particles to decrease. The authors ascribed the decrease in the ζ-potential to
the heat-induced precipitation of calcium phosphate at higher pH on heating. Calcium phosphate can shield the negatively charged casein micelles and reduce the $\zeta$-potential (Anema & Klostermeyer, 1997a; Kudo, 1980).

The pH-dependent dissociation of casein micelles over a wider range of temperatures from 20 °C to 120 °C has also been examined. At pH > 6.7, it was observed that heat treatment as low as ~ 40 °C was able to initialize the dissociation of micellar caseins. In addition, the dissociation rate was greatest within the first 5 min of heating, with little further change on prolonged heating (up to 20 min). The percentage of the dissociation of individual caseins at a given pH was as follows: $\kappa$-casein $>$ $\beta$-casein $>$ $\alpha$-casein (Anema, 1998; Anema & Klostermeyer, 1997b; Anema & Li, 2000).

### 2.6.2.2 Heat effects on whey proteins

Applying heat treatment at > 70 °C in milk can irreversibly denature whey proteins and result in their aggregation. These heat-induced aggregates in milk have drawn much attention due to their profound technological effects on milk functionalities (Donato & Guyomarc'h, 2009).

De Wit (1990) described the thermal denaturation of whey proteins as two successive processes. The denaturation starts with the native protein molecules unfolding to expose side chain groups that are originally buried in the native structure. In the
second step, the unfolded protein molecules aggregate with each other through disulphide bonds, hydrophobic interactions and ionic linkages. These aggregates are mostly β-Lg/β-Lg aggregates. The rate of thermal denaturation of α-Lac and β-Lg was studied (Dannenberg & Kessler, 1988c; Dewit & Swinkels, 1980; Hillier & Lyster, 1979; Oldfield, Singh, Taylor, & Pearce, 1998; Roefs & De Kruijff, 1994). In addition to the formation of whey protein aggregates, unfolded whey protein molecules also associate with κ-caseins through disulphide bonds to form whey protein/κ-casein aggregates on the surface of the casein micelles (Noh, Richardson, & Creamer, 1989; Singh & Creamer, 1991; Smits & Van Brouwershaven, 1980).

Law and Leaver (2000) showed that the aggregation rate of β-Lg and α-Lac significantly increased with the increase of the initial pH (studied pH range from 5.2 to 8.8) when skim milk was heated at 80 °C. Studies using model solutions of β-Lg also showed that the rate of formation of heat-induced aggregates increased as pH increased 6.0 to 8.0 (Hoffmann, Sala, Olieman, & De Kruijff, 1997; Hoffmann & Van Mil, 1997). Hoffmann and Van Mil (1997) explained the higher aggregation rate of β-Lg at higher pH by showing the reactivity and accessibility of the free thiol of β-Lg was higher at higher pH. The increased conversion of the thiols into disulphide bonds at higher pH contributes to the intermolecular binding between β-Lg, or β-Lg and κ-caseins.

The pH values prior to heating of milk can also affect the location of the whey
protein/κ-casein aggregates. Various studies have shown that the higher the pH prior to heat treatment, the less whey proteins are associated with the κ-caseins on the micelle surface. For example, Smits and Van Brouwershaven (1980) applied heat treatment (90 °C/20 min) to a mixture of whey proteins and casein micelle at pH 5.8, 6.3, 6.8 and 7.3 and they observed that 83 %, 76 %, 44 % and 24 % of β-Lg sedimented with casein micelles after ultracentrifugation. Anema and Li (2003) observed that ~ 70 %, 60 %, 51 %, 45 %, and 30 % of whey proteins were associated with the casein micelles, when heat treatment (90 °C/30 min) was applied to skim milk at pH 6.50, 6.55, 6.60, 6.65, and 6.70, respectively. Similar results were reported by other authors (Anema & Klostermeyer, 1997b; Anema & Li, 2003b; Oldfield, Singh, Taylor, & Pearce, 2000; Vasbinder & De Kruif, 2003).

It is generally accepted that these results are due to the higher dissociation rate of the micellar κ-caseins at higher pH during heat treatment (Anema, 1998, 2007 & 2008; Anema & Klostermeyer, 1997b; Anema & Li, 2000; Oldfield, et al., 2000; Singh & Fox, 1985 & 1986). At the higher pH, more κ-caseins dissociate from the micellar phase into the serum phase during heat treatment. Denatured whey proteins then associate with the κ-caseins in the serum phase and form whey protein/κ-casein aggregates. Hence there is less aggregation on the micelle surface (Anema & Li, 2003b). However, there is no evidence to rule out the possibility that, on heating at higher initial pH, denatured whey proteins associate with the κ-caseins on the micelle surface and contribute to the subsequent dissociation of κ-caseins. The whey
protein/κ-casein aggregates may then detach from the micelle surface into the serum phase as a whole (Guyomarc'h, 2006).

The pH values prior to heating also affect the size of the heat-induced aggregates. Using size exclusion chromatography, Donato and Dalgleish (2006) showed the size of the heat-induced aggregates in serum phase decreases with increase in pH (6.3 – 7.3) prior to the heating (90 °C/10 min) of skim milk. Using size exclusion chromatography, Renan et al. (2006) observed that the heat-induced aggregates of milk at pH 6.5 had larger size than those at pH 7.2. This may be due to the higher pH promoting the denaturation and aggregation of the whey proteins and thus the reaction is terminated faster and smaller aggregates are formed (Guyomarc'h, 2006).

2.6.3 Pulsed electric fields (PEF) treatment

2.6.3.1 PEF processing technology

PEF is an innovative processing technology for liquid food preservation. It can be used for pasteurization of pumpable foods at low or moderate temperatures. In PEF processing, foods are placed between two electrodes in a treatment chamber and a short burst of high voltage (in the order of 10 to 80 kV) is applied to the foods. Due to the presence of ions, the electrical current can be transferred within the liquid foods. Consequently, the applied electrical energy destroys the bacterial cell membrane causing microbial inactivation (Barbosa-Cánovas et al., 1999). In addition, PEF
processing is capable of inactivating enzymes (Barbosa-Cánovas & Bermudez-Aguirre, 2010; Ho, Mittal, & Cross, 1997). Because the electrical energy is applied in the form of short pulses, the conversion of the electrical energy to heat is insignificant and the processing temperature can be controlled near room temperature. Hence the organoleptic and nutritional properties of the foods are retained, while the microbiological quality is ensured (Barbosa-Cánovas & Bermudez-Aguirre, 2010). Moreover, the treatment time of PEF is only microseconds, so the energy loss due to heating the food is minimal. From an energy consumption point of view, PEF processing requires less energy to obtain a microbial safety level in foods, particularly in a continuous processing system, compared to traditional thermal processing (Barbosa-Cánovas et al., 1999). PEF processing technology has attracted considerable attention from the food industry and it is considered to be a promising alternative to traditional thermal processing. PEF processing have been successfully applied to electrically conductive foods with low viscosity such as milk, soymilk, pea soup, liquid egg and orange, apple, tomato and cranberry juices (Gauri, 2005).

2.6.3.2 PEF processing systems

A PEF processing system is comprised of a high-voltage (HV) pulse generation system, a treatment chamber, a fluid handling system as well as temperature monitoring and controlling devices (Barbosa-Cánovas & Altunakar, 2006). A flow chart of a continuous PEF processing system with basic components is shown in Figure 2.8.
Generation of electric pulses

The HV pulses are transferred to the PEF processing system via an HV pulse generator at required intensity, shape and duration. A pulse generator normally consists of a HV power supply, an energy storage tank (which is normally comprised of capacitors and electrical insulators) and a switching device.

![Figure 2.8 Flow chart of continuous PEF processing system with basic components. Adapted from Barbosa-Cánovas et al. (1999).](image)

Pulse characteristics

The pulse width, waveform and frequency are determined by the specification of the storage tank and the switching device. The pulse waveform most commonly applied is the square wave. It provides a relatively constant peak voltage over the whole pulse width, which generates an effective high electric field (Zhang, 1995). To achieve this kind of pulse, the capacity of the energy storage tank is required to be orders of magnitude larger than the energy for one pulse. By controlling a switching device that
can be turning on and off hundreds to thousands of times per second, multiple square pulses can be generated. The pulse width and the pulse frequency can be regulated by this kind of switching device (De Haan, 2007). When two of this kind of circuit are combined, bi-polar pulses can be obtained. The shapes of these pulses are shown in Figure 2.9.

![Figure 2.9 Different waveforms of PEF pulses: (a) Square wave form. (b) Bi-polar square wave form.](image)

**Treatment chamber**

PEF treatment chambers are designed to house the discharging electrodes to transfer the electrical energy to solid or pumpable foods held or passed through the chambers. A treatment chamber consists of two electrodes separated by insulating material forming an enclosure for food contact (Zhang, 1995). Changes in the chamber geometry and configuration can have a marked effect on the flow, the electric field distribution as well as the temperature distribution of the food during PEF processing.
Thus the treatment chamber design is a key element for effective PEF processing. The design of the treatment chambers has evolved over the last decades. Static treatment chambers for batch processing were designed and used in the earlier studies. The pioneer research groups include Sale and Hamilton (1967), Dunn and Pearlman (1987), Grahl et al. (1992). More recently, continuous treatment chambers have been developed for the commercialization of PEF processing. Processed foods are able to be pumped through the chambers in a continuous PEF processing system, which can significantly increase the efficiency of production. In a continuous PEF processing system, parallel plate chambers, co-axial flow chambers and co-field flow chambers are the important designs (Figure 2.10). Among those designs, co-field flow chambers are most commonly used for PEF processing because this design reduces the chance of electrode erosion and local bubble formation (Fox, Esveld, & Boom, 2007). In a co-field chamber, two metal pipes are used as the electrodes, which also serve as the entrance and the exit for the foods pumped through the system. Two or more of the co-field chambers are usually connected in series and assembled in a flow cell to form a multiple-chamber system to maximize the PEF effects (Van Den Bosch, 2007). Co-field treatment chambers were used in this study.
Figure 2.10 Different continuous PEF treatment chamber designs. (a) Parallel plate chamber (adapted from Zhang et al. 1995). (b) Co-axial flow chamber (adapted from Zhang et al. 1995). (c) Co-field flow chamber (adapted from Zhang et al. 1995). (d) Co-field flow chambers (adapted from Li et al. 2005).

2.6.3.3 PEF effects on milk proteins

PEF effects on casein micelles

Floury et al. (2006) reported that PEF treatment (45 to 55 kV/cm field strength, 40 to 120 Hz, energy input 0 to 100 kJ/kg, outlet temperature < 30 °C) of raw skim milk
can reduce the size of casein micelles. However, a recent study by Hemar et al. (2011) showed that the reduction of particle size of PEF treated milk (at natural pH) and milk concentrates (at natural pH) was due to the high shear exerted on the samples when pumped through the processing system rather than the electrical effects of PEF processing.

**PEF effects on whey proteins**

Barsotti et al. (2002) applied PEF (electric field strength up to 30 kV/cm, 200 exponential decay pulses at 1 Hz) to 2 % β-Lg solutions and did not observe marked unfolding of the β-Lg molecules. They increased the concentration of the β-Lg solution to 8 – 12 % for PEF processing to detect if there were aggregates, but found no evidence of protein aggregation using PAGE techniques. Li et al. (2005) investigated the PEF processing effects (41.1 kV/cm field strength, 54 µs with bipolar pulses, outlet temperature 43.8 °C) on the secondary structure of IgG in solutions. No changes in its secondary structure or thermal stability were detected. Sui et al. (2011) showed that PEF processing (30 kV/cm field strength, up to 211 µs treatment time, 131.9 kJ/L energy input) of whey protein isolate (WPI) solutions did not change the physicochemical properties of the protein (protein aggregation, surface hydrophobicity, exposed sulphhydryl groups and thermal stability). On the other hand, Perez and Pilosof (2004) found that PEF processing (12.5 kV/cm field strength, 2 ms treatment time, 1631 J/mL energy input, sample temperature after processing < 35 °C) of β-Lg solutions can denature β-Lg and induce partial aggregation of the proteins.
They proposed that PEF processing can polarize the protein molecules and expose the hydrophobic and thiol groups that were originally buried in the molecules, leading to protein aggregations through hydrophobic and thiol/disulphide interactions. It should be noted that the PEF processing system (Gene Pulser II Electroporation System, Bio-Rad) in their study was designed for introducing macromolecules into cells rather than for food processing. Xiang et al. (2010) applied PEF (12 to 20 kV/cm, 10 to 20 pulses, sample temperature < 35 °C after treatment) to WPI solutions in a food processing PEF system (batch parallel plate treatment chamber). Their results indicated that the polarity of the tryptophan residues microenvironment in whey proteins increased and also that there were more hydrophobic regions on the surface of the protein molecules, which was in agreement with the proposed mechanism by Perez and Pilosof (2004) (see above).

The reason why various studies on PEF processing of milk proteins present contrasting results may be due to the fact that a wide variety of processing systems and conditions were used in different studies (Table 2.6). In particular, the PEF treatment chamber design is one of the key factors governing the PEF effects on processed samples. In addition, there is often insufficient detail on the processing parameters and conditions in many published papers, which makes comparisons between different studies very difficult. Complete PEF processing conditions and parameters should always be given when reporting PEF processing effects.
Table 2.6 PEF processing effects on milk proteins in various studies.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Processed sample</th>
<th>PEF conditions</th>
<th>Effects on proteins</th>
</tr>
</thead>
</table>
| Floury et al., 2006 | Raw skim milk               | System: Continuous (5 L/h)  
Pulse shape: Square  
E-strength: 45 or 55 kV/cm  
Pulse width: 0.5 or 0.25 µs  
Frequency: 40-120 Hz  
Treatment time: 2.13 or 3.35 µs  
Specific energy: 45-82 kJ/kg  
Temperature: < 50 °C | Decrease in casein micelle size  
Decrease in renneting time  
and increase in rennet-induced curd firmness |
| Hemar et al., 2010 | Skim milk or milk concentrate | System: Continuous (14.4 L/h)  
Pulse shape: Square bi-polar  
E-strength: 45 kV/cm  
Pulse width: 2 µs  
Frequency: 1370 Hz  
Treatment time: 20 µs  
Specific energy: 202 kJ/kg  
Temperature: 30 °C | No effects on proteins due to pulsed electric fields |
| Barsotti et al., 2002 | β-Lg solutions (2%, 8% or 12%). | System: Batch  
Pulse shape: Exponential decay  
E-strength: 31.5 kV/cm  
Pulse width: Not mentioned  
Pulsed number: 1, 32, 64  
Frequency: 1Hz  
Treatment time: Not mentioned  
Specific energy: Not mentioned  
Temperature: < 30 °C | Sulphydryl groups became more reactive but the change reversed in 30 min. |
| Li et al., 2005 | IgG solutions                | System: continuous (3.6 L/h)  
Pulse shape: Square bi-polar  
E-strength: Not mentioned  
Pulse width: Not mentioned  
Frequency: Not mentioned  
Treatment time: Not mentioned  
Specific energy: Not mentioned  
Temperature: Not mentioned | No changes in the secondary structure |
| Sui et al., 2011 | WPI solution                | System: Continuous (3.6 L/h)  
Pulse shape: Square bi-polar  
E-strength: 35 kV/cm  
Pulse width: 2 µs  
Frequency: 100 Hz  
Treatment time: 19.2 µs  
Specific energy: 131.9 kJ/kg  
Temperature: < 30-75 °C | No changes in protein aggregation, surface hydrophobicity, sulphydryl groups or thermal stability.  
Decrease in heat-induced gel strength of the protein solution. Increase in the gelation time of the sample |
<table>
<thead>
<tr>
<th>Authors</th>
<th>System</th>
<th>Pulse Shape</th>
<th>E-strength</th>
<th>Pulse width</th>
<th>Frequency</th>
<th>Treatment Time</th>
<th>Specific Energy</th>
<th>Temperature</th>
<th>Induced Physical Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perez and Pilosof, 2004</td>
<td>Electroporation system</td>
<td>Exponential decay</td>
<td>12.5 kV/cm</td>
<td>Not mentioned</td>
<td>Not mentioned</td>
<td>2000 µs</td>
<td>1631 kJ/kg</td>
<td>&lt; 35 °C</td>
<td>Induced partial aggregation</td>
</tr>
<tr>
<td>Xiang et al., 2010</td>
<td>Batch</td>
<td>Exponential decay</td>
<td>12-20 kV/cm</td>
<td>Not mentioned</td>
<td>0.5 Hz</td>
<td>Not mentioned</td>
<td>Not mentioned</td>
<td>Not mentioned</td>
<td>Increase in the gelation rate of the protein solution.</td>
</tr>
</tbody>
</table>

**2.6.4 Ultrasound (US) treatment**

Ultrasound refers to the sound waves that are above the human hearing detection (> ~ 20 kHz). In the food industry, low intensity ultrasound (< 1 W/cm²) at high frequency (5-10 MHz) is typically used for non-invasive detection and characterisation of the physical properties of food materials (e.g. texture, viscosity, composition). On the other hand, high intensity ultrasound (10-1000 W/cm²) at low frequency (20-100 kHz) has been used to chemically or physically modify food materials (Patist & Bates, 2008; Suzuki, Lee, Padilla, & Martini, 2010). Dating back to the 1950’s, the first high intensity ultrasound application in food industry was in equipment cleaning and homogenization (Knoerzer, 2007). Since then high energy ultrasound processing has been explored for many other applications in food industry such as extraction,
crystallization, filtration, separation, defoaming, particle size reduction, viscosity alternation, and enzyme and microbial inactivation (Knoerzer, 2007; Patist & Bates, 2008).

2.6.4.1 US processing principles

Propagation of ultrasound waves in liquid medium

When ultrasound is radiated into a medium, the medium will respond to the propagation of the sound waves by vibrating elastically. There are two forms of the medium vibration: condensation and rarefaction. During condensation, the particles in the medium are compressed together thus the space between particles is condensed. Consequently, the pressure and density of the medium increase. During rarefaction, the particles in the medium are driven apart causing a decrease in the pressure and density of the medium. Therefore, pressure (also referred as acoustic pressure) variation is created in the medium (Bermúdez-Aguirre, 2011). However, most liquid media are not very elastic and compressible. Hence, if the variation of the acoustic pressure is great enough in the liquid medium, the liquid can be literally “torn apart” and microbubbles of gas and vapour will form to relieve the pressure created by the sound waves (Kentish, 2011). The formation, growth and implosion process of these microbubbles is called cavitation, which is the major mechanism responsible for most applications of ultrasound in food industry.
Cavitation

Under the influence of the ultrasound waves, the gas and vapour microbubbles in the liquid medium will oscillate in diameter with the variation of the acoustic pressure. During oscillation the microbubbles expand, accompanying the storage of potential energy in the microbubbles. Over a period of acoustic cycles of expansion, the microbubbles grow to a critical size which will lead to the violent collapse (Ashokkumar et al., 2010; Yasui, 2002). The extremely violent collapse of the microbubbles generates extremely high temperature (5000 K) and pressure (1000 atm) in hot spots, which in turn produces very high shear energy shock waves and turbulence in the cavitation zone (Soria & Villamiel, 2010; Suslick, Hammerton, & Cline, 1986). It has been showed that the bubble size is inversely proportional to the ultrasound frequency (Lorimer & Mason, 1987). Therefore, ultrasound with lower frequency can lead to larger cavitation microbubbles, resulting in higher temperature and pressure which in turn generate stronger shock waves and turbulence. For example, Figure 2.11 shows that at a given power intensity, the cavitation bubble generated from 40 kHz US processing has a larger radius compared to that generated from 850 kHz US processing.
Figure 2.11 The size of the cavitation bubble generated from 850 kHz or 40 kHz at different power intensity. Adapted from Holland and Apfle (1998).

Physical effects

As described above, the violent collapse of these microbubbles generates shockwaves and streaming in the cavitation zone and these are referred to as the physical forces of the cavitation. The oscillation of the microbubbles will generate pressure fluctuation in the surrounding leading to the generation of turbulence on a micro-scale called cavitation microstreaming (Kentish, 2011). Besides these physical forces generated by cavitation microbubbles, the propagation of ultrasound waves cause variation of pressure in the medium, which will lead to the strong vibration of the medium. This kind of mechanical vibration is the non-cavitation physical force. All the phenomena described above are known collectively as the physical forces of ultrasound.
processing (Bermúdez-Aguirre, 2011; Mason, 2003).

**Chemical effects**

The collapse of the microbubbles occurs in a time less than 1 µs and consequently generates near-adiabatic heat up to 5000 K in the bubbles (Suslick et al., 1986). Pressure (1000 atm) is also generated during this event. In an aqueous medium, gas molecules (mainly water vapour H$_2$O) will diffuse into the microbubbles during the bubble growth in ultrasound processing. When these molecules are subjected to the extremely high temperature and pressure created by the bubble collapsing, endothermic chemical reactions will occur causing the hemolytic cleavage of the molecules to highly reactive radicals (e.g. OH$^\cdot$, H$^\cdot$) (Ashokkumar et al., 2008). The reactions describing the generation of radicals and new-formed molecular products are shown as below:

\[
\begin{align*}
\text{H}_2\text{O} & \rightarrow \text{OH}^\cdot + \text{H}^\cdot \\
\text{H}^\cdot + \text{H}^\cdot & \rightarrow \text{H}_2 \\
\text{OH}^\cdot + \text{OH}^\cdot & \rightarrow \text{H}_2\text{O}_2
\end{align*}
\]

The number of reactive radicals generated during cavitation depends on both the cavitation temperature and the number of the microbubbles generated at a given frequency and specific energy level. The number of microbubbles is the dominant factor (Kentish, 2011). At low frequency (e.g. 20 kHz), the size of the microbubble
can grow up to 100 µm and generate a larger amount of heat, compared to that generated at higher frequency (Yasui, 2002). However, there are more radicals generated at a higher frequency range (200 – 500 kHz) (which contribute to greater chemical effects) because the total number of the microbubbles generated in a given liquid volume is larger than that generated at lower frequency (Ashokkumar, Lee, Kentish, & Grieser, 2007). The OH· generated from cavitation have been used to oxidize organic pollutants (Inazu, Nagata, & Maeda, 1993), while H· have been used to reduce metal ions to generate metal nano-particles (Hyeon, Fang, & Suslick, 1996).

It has also been shown that OH· can be used to increase the antioxidant activity of foods by enhancing the degree of hydroxylation in food materials (Ashokkumar, et al., 2008).

**2.6.4.2 US processing systems**

There are three main components in an ultrasound processing system:

**Electrical power generator**

It is the energy source of the ultrasound processing system.

**Transducer**

It is a key device in the ultrasound system that converts electrical energy into ultrasonic energy by vibrating mechanically at ultrasonic frequencies (Mason, 1998). The piezoelectric transducer, which was used throughout this project, is the most
commonly used type due to its efficiency in energy conversion (Leadley & Williams, 2002; Mason, 1998).

**Emitter**

After the electrical energy is converted to ultrasonic energy by the transducer, the ultrasonic energy is then transmitted to the processed samples (mostly liquid medium) by the emitter (Mason, 1998). There are a number of commonly used ultrasound processing emitters including: **ultrasonic bath**, **ultrasonic probe**, and **transducer-plate**. In the **ultrasonic bath** the transducers are attached to the underside of a tank and the transducers radiate ultrasound waves directly into the samples held in the tank. For the **ultrasonic probe**, a horn emitter is attached to the transducer to amplify the ultrasonic signal. In a **transducer-plate**, the transducers are completely encapsulated in stainless steel and are submersed into the processed medium to function as the emitter.

### 2.6.4.3 US processing effects on milk proteins

Numerous studies have showed that US processing can change the physicochemical properties and functionalities of milk proteins. Most of these studies were conducted in protein solutions rather than in milk. The applications of ultrasound in dairy processing have been reviewed by several different authors (Ashokkumar *et al.*, 2010; Bermúdez-Aguirre, 2011; Villamiel *et al.*, 1999).
US effects on casein micelles

Taylor and Richardson (1980) reported that sonification reduced the turbidity of skim milk. They hypothesized that sonification disrupts the larger casein micelles into smaller casein micelles, but does not fully disrupt the casein micelles into freely soluble individual casein proteins. However, they did not specify the frequency, power intensity or energy input used in the processing. Villamiel and de Jong (2000) processed raw milk in a continuous US processing system (20 kHz, 150 W power, 11 – 28 mL/min flow rate) with (55 – 75.5 °C) or without heat and they observed no change in the total content of caseins. According to the given power input and flow rate, the specific energy input of the US processing was calculated as 818 – 321 kJ/kg. However, the given processing power 150 W is the maximal power of the process unit instead of the actual power during processing. Thus the calculated specific energy is largely overestimated and the actual specific energy during processing remains unknown. Nguyen and Anema (2010) applied 22.5 kHz US (50 W for 0 – 30 min in 18 g of sample, calculated specific energy 0-5000 kJ/kg, with or without temperature control) on skim milk. With the processing temperature below 70 °C, authors observed a slight reduction (~ 15 – 20 nm) in casein micelle size and some dissociation of κ-caseins into the serum phase. The results of these studies suggest that US processing can disrupt casein micelles in milk. The authors reported that the US processing had negligible effects on acid-induced gelation behaviour and no further investigation was carried out to explore the US effects on casein micelle dissociation.
In order to demonstrate the US processing effects on casein micelles in a less complicated medium, Madadlou et al. (2009a) re-assembled caseins into micelle particles in phosphate buffer and then applied US treatment. They reported the disruption of re-assembled micelle particles by 35 kHz US processing (0, 2, 4.1 and 6.6 W for 6 hr to 100 mL sample, calculated specific energy 0, 432, 886 and 1426 kJ/kg) with processing temperature control at ~ 30.5 °C. The disruption was greater when the pH of the caseinate solutions was higher, and this was attributed to the looser structure of micelle particles at higher pH. Moreover, the size reduction of the micelle particles increased with the increase in processing power due to the higher cavitation effect at higher processing power level. Madadlou et al. (2009b) demonstrated that disruption of re-assembled micelle particles was more effective at 130 kHz than 35 kHz. They suggested this was due to the higher chemical effects in US processing at 130 kHz (Madadlou, Mousavi, Emam-Djomeh, Ehsani, & Sheehan, 2009b). In a recent study, Madadlou et al. (2012) further demonstrated that applying dual-frequency (130 kHz and 24 kHz) US processing to the re-assembled micelle particles can have a synergistic effect to achieve effective particle disruption, compared to the effect of a dual-frequency of 35 kHz and 24 kHz US processing. Despite the significant US effects on the disruption of re-assembled micelle particles, re-assembled micelle particles in these studies are different from casein micelles in milk due to the absence of MCP in these particles, which is an essential component to native casein micellar structure stability. Studies of US effects on native casein micelles have been mostly conducted at low processing frequencies (~ 20 kHz).
(Nguyen & Anema, 2010; Villamiel & de Jong, 2000). Although Madadlou et al. (2009b & 2012) reported that the chemical effects at higher frequency (130 kHz) have greater impact on re-assembled micelle particle disruption compared to 35 kHz US processing, they did not quantify the chemical effects (liberation of free radicals) in both US processing systems. Their conclusion is merely based on the previous report (Ashokkumar et al., 2008) that the quantity of free radicals generated during US processing increases from 20 kHz and reaches the maximum at 358 kHz but again decreases when the frequency is increased to 1062 kHz. The difference in the chemical effects between 35 kHz and 130 kHz US processing is not known. Therefore, further investigation of US effects on casein micelles needs to be carried out in a defined US processing system in terms of physical and chemical effects and in milk where the casein micelles are present in natural state.

**US effects on whey proteins**

Villamiel and de Jong (2000) processed raw milk in a continuous US processing system. They observed that more β-Lg and α-Lac precipitated with the casein fraction at pH 4.6 after US processing and concluded that US processing denatured both whey proteins. They also reported that denaturation of whey proteins was enhanced by combining heat and US treatment. Jambrak et al. (2010) showed that US processing of 10 % α-Lac solutions at 20 kHz (39 – 44 W/cm² power intensity, calculated specific energy 310-701 kJ/kg) and 40 kHz (1-2 W/cm², specific energy input unknown as transducer dimensions were not specified) can significantly reduce the
molecular weight of $\alpha$-Lac examined in SDS-PAGE gels. Gulseren et al. (2007) reported that US processing (20 W/cm$^2$ for 0-90 min to 30 mL sample, calculated specific energy 4558 kJ/kg) of BSA solutions can increase the surface hydrophobicity of the protein molecules. The protein molecules also aggregated through disulphide bonds leading to the increase in particle size in US-treated solutions. Arzeni et al. (2010) also reported that the surface hydrophobicity of whey protein molecules was greatly increased after 20 kHz US processing of WPI solutions (4.27 W for 20 min in 5 mL sample, calculated specific energy 1025 kJ/kg). However, it was also reported that 20 kHz US processing (31 W for 1 – 60 min in 60 mL sample, calculated specific energy 31-1860 kJ/kg, processing temperature at 6 ± 4 °C) of whey protein concentrate (WPC) solutions had little effect on the thermal properties, thiol groups, hydrophobic regions and the secondary structures of the whey proteins (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011).

2.7 Process stress-induced changes to milk protein functionality

2.7.1 Heat effects on milk functionality

Renneting properties of milk

It is well-known that heat treatment of cheese milk can cause severe inhibition of
rennet gel formation and texture defects of the curd. The formation of whey protein/κ-casein aggregates on the micelle surface can hinder the attack of rennet on κ-caseins and largely reduce the enzyme activity (Singh & Waungana, 2001; Zoon, 1994). However, heat treatment is still introduced in cheese-making practice to recover whey proteins into cheese curd despite some textural and sensorial defects in the cheese (Banks, 1990; Jameson & Lelievre, 1996; Marshall, 1986). In order to minimize the detrimental effects of heat, combined heat and pH manipulation has been used to incorporate whey proteins into cheese curd (Banks, Law, Leaver, & Horne, 1994; Law, Banks, Horne, Leaver, & West, 1994).

**Acid-induced gelation of milk**

As mentioned in Section 2.5.2, heat treatment of milk can significantly improve the acid-induced gelling properties and these heat-induced effects are shown to be highly correlated to the denaturation of whey proteins (Dannenberg & Kessler, 1988a, 1988b; Lucey, Teo, Munro, & Singh, 1997) and formation of whey protein/κ-casein aggregates (Lucey, Tamehana, Singh, & Munro, 1998).

Some studies showed that higher ratios of serum aggregates to micelle-associated aggregates increased the gelation pH and favoured the gel network development leading to a firmer gel (Anema, Lee, Lowe, & Klostermeyer, 2004; Anema, Lowe, & Lee, 2004; Vasbinder & De Kruijf, 2003). It is suggested that fewer micelle-associated aggregates reduce the steric hindrance of the interaction between casein micelles.
Thus it favours the gelation (Donato & Guyomarc’h, 2009).

In contrast, others found that higher proportions of micelle-associated aggregates induced higher gel firmness (Lucey et al., 1998; Schorsch et al., 2001). However, these samples were prepared by reconstituting the separately-treated whey proteins and casein micelles in the milk ultrafiltrate. The whey proteins were heat-treated in a mineral environment that is different from that in milk. This approach also leads to the different total amount of heat-induced aggregates in the samples. Thus it is difficult to compare these results (Lucey et al., 1998; Schorsch et al., 2001) with those obtained from done by varying pH in milk (Anema, Lee, Lowe, & Klostermeyer, 2004; Anema, Lowe, & Lee, 2004; Vasbinder & De Kruif, 2003). Vasbinder et al. (2003) proposed that the total amount of whey protein/κ-casein aggregates is the main factor influencing the acid gelation rather than the location of those aggregates.

**Heat stability of milk**

The heat stability of milk is a key parameter in sterilization of recombined concentrated milk because of the partial or complete coagulation of the products during sterilization or during storage (O’Connell & Fox, 2003). Singh and Creamer (1991) and Williams et al. (2008) showed that different characteristics of the heat-induced protein aggregates in milk can affect the heat stability of recombined concentrated milk. In addition to the heat-induced protein aggregates, Ca activity plays a crucial role in the heat stability of milk and Augustin and Clarke (1990),
2.7.2 PEF effects on milk protein functionality

Floury et al. (2006) observed a reduction in the viscosity of raw skim milk after PEF processing. They proposed that PEF modification of the hydrodynamic volume of the casein micelles and/or the mineral balance led to the viscosity change. In addition, they observed an increase in rennet curd firmness and a decrease in renneting time in PEF treated milk, compared to non-PEF treated milk. This may be due to the reduction in the micelle size which increased the total micellar surface area and thus increased the rennet reactivity. Yu et al. (2009) applied PEF processing (20 to 30 kV/cm field strength, 2 µs pulse width, 40 to 120 pulses) with heat (35 to 50 °C) or without heat (20 °C) on raw milk. In contrast to the results of Floury et al. (2006), they observed the rennet-induced gel firmness was lower and gelation time was higher in PEF treated milk with or without heat, compared to that of non-PEF treated raw milk. PEF-treated milk has been reported to show higher rennet-induced gel firmness and lower gelation time than pasteurized milk (63 °C/30 min) (Yu, Ngadi, & Raghavan, 2009). However, no analysis of the physicochemical properties of casein micelles or whey proteins was reported by these researchers. Thus the reasons why the rennet-induced gel firmness decreased and gelation time increased were not clear. Recently, Yu et al. (2012) studied the proteolysis profiles (peptide and free amino acid
concentration) of cheese slurry made from raw milk, PEF treated milk and pasteurized milk. The results showed that the proteolytic properties of milk treated by PEF were intermediate between those of raw milk and those of pasteurized milk. These authors concluded that PEF processing had the potential to produce microbial-safe cheese milk without compromising the natural characteristics of the milk due to heat treatment (Yu et al., 2012). Perez and Pilosof (2004) reported that PEF processing of β-Lg solutions enhanced the gelation rate of β-Lg. However, Sui et al. (2011) reported that PEF processing of WPI solutions markedly decreased the heat-induced gel strength and increased the gelation time of the WPI.

### 2.7.3 US effects on milk protein functionality

Tabatabaie et al. (2009) showed yoghurt gels made from 20 kHz US treated skim milk had a microstructure with more interconnected chains, a more smooth and regular surface and more homogenous porosity due to the decrease of casein micelle size, compared to those made from non-US treated skim milk. However the power and energy input were not specified. Madadlou et al. (2010) reported that the application of dual-frequency (130 kHz and 24 kHz, 20.9 W for 0, 60 or 120 min to 100 mL sample, calculated specific energy 0, 752.4 or 1504.8 kJ/kg) US processing on the re-assembled casein micelle solutions improved the elasticity of the acid-induced gels from the US treated caseinate solutions. The gel network was more compact with smaller non-distinguishable particulates (Madadlou et al., 2010). This is most likely
due to the disruption of micelle particles by the US processing. On the other hand, Nguyen and Anema (2010) reported that US processing (22.5 kHz, specific energy 0-5000 kJ/kg) of skim milk with temperature control (< 70 °C ) did not affect the acid-induced gelation properties, despite a reduction in micelle particle size (15-20 nm). There are no reports of the US effects on other casein micelle-related functionalities such as rennet-induced gelation properties or heat stability. A few studies have reported that yoghurt gels made from US treated milk had a better microstructure and texture (Riener, Noci, Cronin, Morgan, & Lyng, 2009 & 2010; Wu, Hulbert, & Mount, 2000). However, these studies were focusing on the US homogenizing effects on fat globules rather than on casein micelles or whey proteins.

Jambrak et al. (2010) reported that US processing of 10% α-Lac solutions at 20 kHz (39 – 44 W/cm² power intensity) and 40 kHz (1-2 W/cm²) increased the solubility (for 20 kHz treated samples), foam capacity and stability (for 20 kHz and 40 kHz treated samples). These effects are most likely due to the US modification of the whey protein molecular structures. Indeed, US modifications of whey protein functionalities have been widely reported. They include breaking down of heat-induced whey protein aggregates and preventing their reformation (Ashokkumar et al., 2009); increasing WPC film tensile strength (Banerjee, Chen, & Wu, 1996) or WPC and WPI gel strength (Arzeni et al., 2012; Zisu, Bhaskaracharya, Kentish, & Ashokkumar, 2010; Zisu et al., 2011); reducing the particle size in WPI solutions (Gordon & Pilosof,
reducing the viscosity of WPC and milk protein retentate solutions (Zisu et al., 2010); reducing the turbidity of whey protein suspension (Martini, Potter, & Walsh, 2010) and WPC and WPI solutions (Zisu et al., 2011) and increasing the solubility of WPC and WPI solutions (Jambrak, Mason, Lelas, Herceg, & Herceg, 2008; Kresic, Lelas, Jambrak, Herceg, & Brncic, 2008). However, Jambrak et al. (2011) reported a decrease of solubility in US treated WPC and WPI solutions in a different US processing set-up (30 kHz) (Jambrak et al., 2011).

Most of the US processing studies were conducted using US at low frequency, usually at 20 to 40 kHz which is the frequency range that can provide the strongest cavitation physical effects (Ashokkumar et al., 2010). Few studies used medium frequency US processing. Jambrak et al. (2008 & 2010) conducted US processing of different whey protein solutions at 20 kHz, 40 kHz and 500 kHz frequency. They reported that 500 kHz US treatment did not have significant effects on the protein structure or functionality in terms of solubility and foaming ability, while 20 kHz and 40 kHz US treatments had great effects on these whey protein properties. However, in these studies, the power and the processing set-ups were different at different processing frequencies (Jambrak et al., 2008; Jambrak, Mason, Lelas, & Kregic, 2010). So it is not valid to compare the frequency effects on the samples in these studies. Comparison of US effects between frequencies should employ the same specific energy input during processing.
Chapter 3

Experimental methodology

Summary

To investigate the effects of PEF processing or US processing on milk, various analytical techniques were used to study the physicochemical and functional properties of milk proteins. In this chapter, the milk preparation will be demonstrated, followed by the description of different analytical methods to study the particle characteristics in milk, the physicochemical properties of proteins in the milk serum phase and the functionality of the milk proteins. In addition, the milk processing experimental designs using traditional heat treatment, PEF treatment or US treatment are specified.

3.1 Milk samples

3.1.1 Milk powder characterisation

Low-heat skim milk powder was purchased from Tatura Milk Industries Limited (Tatura, VIC 3616, Australia). The powder was manufactured in June 2009. The milk powder was vacuum-sealed in bags and stored at 4 °C until the experiments were conducted. The same batch of milk powder was used throughout this study. The milk
powder was characterised for moisture, fat content, total nitrogen, non-protein nitrogen, non-casein nitrogen, casein content, whey protein content, lactose content, calcium, phosphorus (Table 3.1) in Dairy Technical Services Limited, North Melbourne, VIC 3051, Australia.

Table 3.1 Chemical characterisation of skim milk powder

<table>
<thead>
<tr>
<th>TEST</th>
<th>RESULTS</th>
<th>METHOD N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>4.0 % m/m</td>
<td>MOIS 01 12.99</td>
</tr>
<tr>
<td>Fat</td>
<td>0.65 % m/m</td>
<td>FATS 01 12.99</td>
</tr>
<tr>
<td>Total Nitrogen</td>
<td>5.26 % m/m</td>
<td>PROT 01 02.01</td>
</tr>
<tr>
<td>Protein (TN x 6.38)</td>
<td>33.6 % m/m</td>
<td>PROT 01 02.01</td>
</tr>
<tr>
<td>Non Protein Nitrogen</td>
<td>0.318 % m/m</td>
<td>NITF 01 03.03</td>
</tr>
<tr>
<td>Non Casein Nitrogen</td>
<td>1.016 % m/m</td>
<td>NITF 02 03.03</td>
</tr>
<tr>
<td>Whey Protein (WPN x 6.38)</td>
<td>4.45</td>
<td>NITF 03 03.03</td>
</tr>
<tr>
<td>Casein (CN x 6.38)</td>
<td>27.1 % m/m</td>
<td>NITF 03 03.03</td>
</tr>
<tr>
<td>Lactose monohydrate</td>
<td>54.7 % m/m</td>
<td>LACT 02 04.93</td>
</tr>
<tr>
<td>Calcium</td>
<td>1.2 % m/m</td>
<td>MCP 01 04.06</td>
</tr>
<tr>
<td>Phosphorus (as PO4)</td>
<td>3.0 % m/m</td>
<td>PHOS 01 04.93</td>
</tr>
</tbody>
</table>

3.1.2 Preparation of milk

A stock solution of reconstituted skim milk (20 % total solids (TS)) was made by reconstituting low-heat skim milk powder in MilliQ water. The milk was then stirred continuously for 5 h at room temperature (~22 °C ± 3 °C) to ensure the powder was well dispersed. Sodium azide (0.02 % w/w) (Sigma-Aldrich Pty Ltd, Castle Hill, NSW 1765, Australia) was added for preservation of the milk. This stock solution (20 % TS) was then stored at 4 °C overnight.

For pH adjusted milks, the stock solution (20 % TS) was diluted with MilliQ water
and the pH was adjusted to desired values by drop-wise addition of 0.1 M NaOH or HCl and a final concentration of 10 % TS was obtained by adding the required amount of MilliQ water. The pH was measured using a PHM93 pH meter (Radiometer Copenhagen, Mt Waverly, Vic, 3149, Australia). Samples were then stored at 4 °C overnight and processed in the next day.

For the preparation of milks with added EDTA (Ethylenediaminetetra-acetic acid tetra-sodium, Sigma-Aldrich Pty Ltd, Castle Hill, NSW 1765, Australia) (10, 20 and 30 mM), appropriate amounts of 200 mM EDTA was added drop-wise under continuous stirring to the stock solution, which was then diluted with MilliQ water and adjusted pH to 6.7 with 0.1 M NaOH or HCl solution to achieve the final milk concentration of 10 % TS. Samples were then stored at 4 °C overnight and processed in the next day.

In Chapter 4, experiments were carried out on milks at pH 6.7, 7.1, 7.5 and 8.0, and milks with 10, 20, 30 mM EDTA at pH 6.7. In Chapter 5, experiments were carried out on milks at pH 6.7, 7.1, 7.5 and 8.0. In Chapter 6, experiments were carried out on milks at pH 6.3, 6.7, 7.1 and 7.5. In Chapter 7, different milks were selected from the previous experimental chapters for further functionality tests.
3.2 Analytical techniques

3.2.1 Characterisation of milk particles

3.2.1.1 Particle size and ζ-potential by Dynamic Light Scattering (DLS)

The particle size and ζ-potential were measured using DLS in a zetasizer (Malvern Zetasizer Nano ZS, Malvern, Worcestershire, UK). Measurements by DLS require extensive dilution of the sample. To ensure that the sample dilution does not affect the integrity of the milk protein aggregate, and that the proteins are maintained in the same ionic environment upon dilution to avoid dissociation of the casein micelle, dilution of the milk samples for DLS measurements were performed in the milk ultrafiltrate (UF). The milk UF was obtained by centrifuging the milk samples centrifuged at 55,000 × g at 25 °C for 90 min (Beckman-Coulter Optima L-90K ultracentrifuge with a type 55.2 Ti rotor, Beckman Instruments Australia Pty Ltd, Gladesville, NSW, 2111, Australia). The high centrifugation speed was chosen to effectively deposit casein micelles as a firm pellet. The supernatant was further filtered using Millipore Centriprep centrifugal filter device (Millipore Corporation, Billerica, MA, 01821 USA) with 10 kDa cut-off membrane at 2,000 × g (Beckman J6-HC centrifuge, Beckman Instruments Australia Pty Ltd, Gladesville, NSW, 2111, Australia) for 30 min at 25 °C and the UF was obtained.
Prior to measurement, milk (5 µL) was diluted in the UF of the corresponding milk (1.5 mL) and equilibrated to 25 °C for 2 min. The particle size or ζ-potential of the milk casein micelles were then measured by DLS using a measurement angle of 173 ° backscatter and a 633 nm laser. A total of 10 measurements were taken for each sample and the results were reported as the average of the last 5 measurements. The intensity average diameter (z-average diameter) of each sample was obtained from a cumulant analysis of each sample’s correlation function, using a serum viscosity of 1 mPa.s and a refractive index 1.343 as reported by Hemar et al. (2011).

3.2.1.2 Particle size distribution by Size exclusion chromatography (SEC)

The particle size distribution in milk was examined by SEC as reported by Williams et al. (2008). Milk (0.5 mL) was diluted 1:1 with elution buffer and applied to the column of Sephacryl S-1000SF (1.6 cm diameter × 98 cm long, cat. # 17-0476-01, lot # 304180, Amersham Bioscience Pty Ltd, Baulkham Hills, NSW, Australia). Milk then eluted with a buffer system of 50 mM NaCl (Sigma, lot # 070M0144V), 20 mM Tris (Sigma, lot # 046k5402), 3 mM CaCl₂ (Unilab, batch # F2D079), 0.2 g/kg NaN₃ (Sigma-Aldrich, lot # MKBP3457V), pH 6.7 at 0.5 mL/min. The column was run by a SCL-10A system controller (Shinadzu, USA MFG Inc.), LC-10AD pump (Shinadzu, USA MFG Inc.), SIL-10AD auto injector (Shinadzu, USA MFG Inc.) and SPD-10A US-VIS detector set at 280 nm. The total running time is 450 min.
3.2.2 Characterisation of milk proteins in the serum phase

3.2.2.1 Preparation of milk serum

To obtain the milk serum, the milk samples were centrifuged at 55,000 \( \times \) g at 25 °C for 1 hr (Beckman-Coulter Optima L-90K ultracentrifuge with a type 55.2 Ti rotor, Beckman Instruments Australia Pty Ltd, Gladesville, NSW, 2111, Australia). Protein fractions from top to the bottom of the centrifuge tube were clear supernatant layer (serum phase), opalescent layer (mainly contains the smallest micelles) and the sediment pellet (micellar phase). The milk serum used in this study refers to the clear milk supernatant layer excluding the opalescent layer.

3.2.2.2 Protein content measurement

The nitrogen content in milk serum (and milk) was measured using a LECO FP-2000 Nitrogen Analyser (LECO Australia Pty Ltd, Castle Hill, NSW Australia) as reported by (Jayani Chandrapala, Augustin, McKinnon, & Udabage, 2010). The nitrogen content was then converted to protein content using a conversion factor 6.38. The protein content in milk serum was presented as the percentage of total protein in milk.
3.2.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate, identify and quantify the individual milk proteins in the samples. Kits and reagents for SDS-PAGE were also purchased from Invitrogen Australia Pty Ltd (Mulgrave, VIC 3170, Australia). Pre-cast 4-12 % acrylamide gels were used and Mark12™ Unstained Standard was used as a standard protein molecular weight ladder. Electrophoresis was performed under reducing and non-reducing conditions.

For SDS-PAGE under reducing conditions, samples (25 µl) were mixed with 10 µL of 4x NuPAGE® LDS (lithium dodecyl sulphate) sample buffer (prepared from 4x concentrate containing 106 mM Tris HCL, 141 mM Tris base, 2 % LDS, 10 % glycerol, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, and 0.175 mM Phenol Red at pH 8.5) and 5 µL of 1 mM DTT (α-dithiothreitol). For SDS-PAGE under non-reducing conditions, samples (25 µL) were mixed with 10 µl of 4x NuPAGE® LDS sample buffer and 5 µl MilliQ water. All samples were heated at 70 °C for 10 min in a heating block (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA, 94404, USA) to ensure complete reduction before loading into gels. Aliquots (10 µL) of the heated samples were applied to the gels. The running buffer was 1x NuPAGE® MES (morpholino ethane sulphonic acid) SDS buffer (prepared from 20x concentrate containing 50 mM MES, 50 mM tris base, 0.1 % SDS, 1 mM EDTA at pH 7.3). 500 µL of NuPAGE® antioxidant (containing 10 %
N,N-dimethylformamide and 20 % sodium bisulfite) was added in the upper chamber of the gel tank before applying voltage. Gels were then run at 200 V for 35 min.

The gels were then stained with the Simply Blue™ SafeStain coomassie blue reagent and destained with distilled water according to the manufacturer manual. Then the gels were photographed using Gbox (UV transilluminator and Gel Imaging System, SynGene, Frederick, MD 21704, USA). The photographs were analysed using ImageQuant TL Version 2005 (Amersham Biosciences Corp, 800 Centennial Avenue, NJ 08855, USA) to obtain the integrated intensities of the bands. The integrated intensities of each band were measured and used to determine the relative proportions of caseins and whey proteins present in the serum phase of the milks.

For work reported in Chapter 6, the pre-cast gels were changed from 4-12 % acrylamide gels to 12 % acrylamide gels. The SDS-PAGE running buffer was changed from MES SDS buffer to MOPS (morpholino propane sulfonic acid) SDS buffer (prepared from 20x concentrate containing 50 mM MOPS, 50 mM tris base, 0.1 % SDS, 1 mM EDTA at pH 7.7). The rest of the procedure remained unchanged. This method was adopted to achieve a better separation of the casein bands as shown in Figure 3.1: The migration distance between 14 to 31 kDa where the α- (23-25 kDa), β- (24 kDa) and κ-casein (19 kDa) bands get separated, is much longer in the 12 % Bis-Tris gel with MOPS running buffer (blue line) compared to in the 4-12 % Bis-Tris gel with MES running buffer (red line).
Figure 3.1 Migration patterns achieved on NuPAGE® gels in different systems from the manufacturer’s documentation. Available on:
3.2.2.4 $^{31}$P NMR (Nuclear Magnetic Resonance)

$^{31}$P NMR is a well-defined technique for investigating diversiform of phosphorus in milk. $^{31}$P NMR experiments were conducted on a Varian Inova 11T NMR spectrometer (Varian (now Agilent Technologies) NMR Instruments, Palo Alto, CA, USA; $^{31}$P operating frequency 202.3MHz) at a temperature of 25 °C. Samples were placed in a 10 mm o.d. NMR tube and a coaxial Wilma d capillary insert (3 mm o.d) was used to hold a solution of methylene diphosphonic acid (Sigma-Aldrich Pty Ltd) (10 mM in D$_2$O). This solution served as a source of lock signal as well as a chemical shift and concentration reference. Composite pulse decoupling was used with the $^1$H-decoupler being gated off during the pulse delay. Chemical shifts were referenced to the methylene diphosphonic acid signal which was set at 18.9 ppm relative to 85 % (v/v) phosphoric acid. Spectra (500 transients) were accumulated using 60° pulses and a 10s cycle time. The spectral width of 12000 Hz was represented by 32786 data points. The spectra were analysed and peak area was integrated using MestRe-C version 2.3 software.

3.2.2.5 Size exclusion chromatography (SEC)

SEC was used to determine the amount of soluble aggregates and monomeric proteins in the milk serum phase. Milk serum was filtered through a 0.2 µm disposable syringe filter unit (Advantech®, Toyo Roshi Kaisha, Ltd, Japan) and applied to a column (TSK Gel G5000 PW; 10 µm, 300 × 7.5 mm) and eluted with buffer (100 mM
ammonium bicarbonate (Sigma-Aldrich Pty Ltd), pH 7.0) at a flow rate of 0.5 mL/min. The column was run by a CBN-20A controller (Shimadzu, USA MFG Inc.), LC-10Ai pump (Shimadzu, USA MFG Inc.) and a SPD-20A variable wavelength monitor (Shimadzu, USA MFG Inc.) set at 280 nm. The total running time for each sample was 34 min.

In order to characterise the soluble aggregates in the serum phase, fractions of the soluble aggregates were collected using FRC-10A fraction collector (Shimadzu, USA MFG Inc.). To obtain enough materials of the fractions for further analysis, each fraction was collected from 4 runs of the same milk serum and evaporated to dryness using a GeneVac concentrator (HT-4 series, GeneVac Technologies). To identify the protein composition in the fractions, the dried fractions were re-dissolved in deionised water to restore the concentration to that of the original protein in fraction collected and applied to SDS-PAGE (reducing conditions) as described above. The collected fraction was also used for measurement of particle size, ζ-potential using the methods described above.

### 3.3 Functionality analysis

In Chapter 7, different milks were selected for functionality tests based on the processing effects from Chapters 4, 5 or 6. Table 7.1 (Chapter 7, Section 7.2.1) listed the selected milks and corresponding functionality tests. It is important to note
that all the selected milks were re-adjusted back to pH 6.7 before the functionality
tests.

In the rheological test which involves the application of oscillatory strain or stress to
the sample, some of the main parameters including G' (storage modulus), G" (loss
modulus) and tan δ (loss tangent) provide important information on the gel formation
process. G' is a measure of the energy stored per oscillation cycle and G" is a measure
of the energy dissipated as heat per cycle. Tan δ is the ratio of the viscous to elastic
properties (G"/G') (Lucey & Singh, 2003).

3.3.1 Rennet-induced gelation

3.3.1.1 Rheological analysis

The rheological properties of rennet-induced gelation were determined by an Anton
Paar-Physica stress control rheometer (MCR 301, Anton Paar Physica, Physica
Meßtechnik GmbH, Stuttgart, Germany) with a cup and bob geometry (CC27/s, Serial
# 1805, diameter 26.661 mm, length 39.985 mm) using low-amplitude dynamic
oscillation measurement. Milks (20 mL) in glass beakers were pre-warmed to 31 °C
in water bath for 10 min. A 3.5 IMCU/mL rennet solution (Chymaz Plus, CHR
Hansen, Hørsholm, Denmark) was prepared by diluting 875 µL stock rennet solution
(200 IMCU/mL) to 50 mL using volumetric flask. 0.2 mL rennet solution (3.5
IMCU/mL) was added to 20 mL pre-warmed milk to achieve a final strength of rennet
0.035 IMCU/mL and stirred for 1 min. The milk-rennet mixture (~17 mL) was then immediately transferred into the cup. The bob was lowered and submerged in the solution. A ceramic cover was placed over the cup to prevent evaporation. The measurements were carried out at a constant frequency of 1 Hz and a constant shear strain of 2.5% at constant temperature 31 °C (Guine et al., 1997). Data points were collected at 30 sec intervals for 60 min. The gelation time was defined as the time at which the storage modulus (G’) first passed above 0.2 Pa. Maximum curd firming rate was defined as the slope of the line drawn through the linear region of the G’ as a function of time graph where G’ increased most rapidly. The curd firmness was defined as the G’ recorded at 60 min of gelation process (Auldist, Mullins, O’Brien, & Guinee, 2001).

3.3.1.2 Confocal laser scanning microscopy (CLSM)

The microstructure of the set rennet-induced gels was examined by CLSM. The fluorescence protein dye, acridine orange (~0.01 %) was added (3 drops) into milk (10 mL) and stirred for 2 min. Milk was then pre-warmed to 31 °C and rennet added (final strength of 0.035 IMCU). After mixing well for 30 sec, the milk-rennet mixture was transferred to an object slide with a cavity. A coverslip was placed to cover the sample. The slide was then placed in an incubator set at 31 °C for 6 hr. The gels were then examined using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany), with a 63 × glycerol immersion HCX PL APO
objective. The fluorescent dye was excited by a 488 nm argon laser and the reflected light detected at 530 – 650 nm. Typical micrographs were reported.

3.3.2 GDL-induced gelation

The rheological properties of GDL-gelation were monitored by an Anton Paar-Physica stress control rheometer, with a cup and bob geometry as described above, using low-amplitude dynamic oscillation measurement. Milk (30 mL) in glass beaker was pre-warmed to 43 °C in a water bath for 10 min. GDL (Gluconic-delta-lactone, Sigma-Aldrich Pty Ltd) (1.3 %) was added to milk and the mixture was stirred for 1 min. The mixture was then immediately sub-sampled (~17 mL) into the cup and the bob was lowered to submerge in the sample. A ceramic cover on the cup was used to prevent evaporation. The measurements were carried out at a constant frequency of 1 Hz and a constant shear strain of 1 % at constant temperature 43 °C. Simultaneously as the sample (~ 17 mL) was transferred into the cup, a pH meter (Hanna Instrument, HI 9318) probe was placed into the rest of the sample (~ 13 mL) in the glass beaker which was incubated in a water bath at 43 °C. The pH of the sample was recorded in a pH/temperature logging software (Hanna instrument HI 9200) through the pH meter. The pH of the GLD-milk mixture was recorded at 1 min intervals and dropped to ~ 4.6 in 3 hr at 43 °C. Accordingly, the oscillation measurement was carried out for 3 hr and data points were collected at 1 min intervals. The gelation time was defined as the point when gels had a $G' \geq 1$ Pa.
the curd firmness was defined as the $G'$ recorded at 3 hr of gelation process. The maximum in $\tan \delta$ was defined as the point after gelation when the value of $\tan \delta$ increased to a maximum (Lucey et al., 1998).

3.3.3 Determination of heat stability of recombined concentrated milk

Reconstituted skim milks (10 % TS) (300 mL) were frozen in freeze-drying flask using liquid nitrogen. Frozen milk was then freeze-dried to skim milk powder using a freeze drying unit (DYNAVAC) with a high vacuum pump (Jayvac, Serial # B27428). Skim milk powder (50 g) was then dissolved in distilled water (184.75 g) at 45 °C with continuous stirring for 2 hr for reconstitution. After removing as much foam as possible, molten anhydrous milk fat (19.99 g) was added to the reconstituted milk concentrate (220 g) to make recombined concentrated milk with a final formation of 18 % solids non-fat : 8 % fat. The recombined concentrated milk was then homogenised (14 MPa) at 45 °C using a homogeniser unit (EmulsiFlex-C3, AVFSIN Inc. 2450 Don Reid Drive, Ottawa, ON, Canada).

Portions of homogenised recombined concentrated milk (22 mL) were adjusted with water or HCl/NaOH (0.2 M) to achieve a range of pH. The pH-adjusted samples (20 mL) were then transferred into screw-capped stainless steel tubes. The tubes were placed in a boiling water bath for 5 min then transferred to a glycerol bath at 120 °C
with continuous rocking for 13 min. Tubes were then immediately transferred to a 25 °C water bath and rocked gently for 1 min to allow the milks to equilibrate to 25 °C. Viscosity was measured using a stress control by using a rheometer (Parr MCR301), with a double gap geometry (DG 267/Ti, serial # 3524). 10 measurement points were obtained at 20 sec intervals using a constant shear rate 50 s⁻¹ at 20 °C.

### 3.3.4 Viscosity of milk during concentration

To concentrate milk by evaporation, milks (300 mL) were transferred into a 2 L round bottom glass flask. The concentration of milk was carried out in a rotary evaporation system using a V-700 vacuum pump (BUCHI, Switzerland), R-124 rotavapor (BUCHI, Switzerland), B-480 water bath (BUCHI, Switzerland), a thermomix 1460 recirculating heated water bath pump (BUCHI, Switzerland) and a frigomix 1497 heated water bath (BUCHI, Switzerland). The evaporation was at constant temperature 60 °C, under ~ 50 mbar vacuum. Evaporation was monitored periodically by weighing the flask and calculating the mass of water removed to predict approximate concentration (% of TS) of the milks. Once a suitable mass of water had been removed, a sample was taken for viscosity measurement. The concentrations of the samples were also verified using a moisture analyser MA 30 (Statorius, Geottingen, Germany).

Viscosity was measured immediately after sampling (within about 1 min of removal
from the rotary evaporator) in a flow measurement using a stress control rheometer (Parr MCR301), with double gap geometry (DG 267/Ti, serial # 3524). 10 measurement points were obtained at 20 sec intervals using a constant shear rate 50 s$^{-1}$ at 20 °C.

### 3.4 High-heat treatment of milk samples

In Chapter 6, the US effects on milk proteins are investigated in high-heat treated milks. The high-heat treatment is as followed: Milks (200 mL) were heat treated at 90 °C for 10 min in a 500 mL glass beaker immersed in a water bath set to 95 °C with continuous rocking. After heat treatment, milks were immediately cooled to room temperature by immersion of the glass beakers in ice water. Heat treated milks were sub-sampled for subsequent US treatment.

### 3.5 Novel processing technologies Pulsed: electric fields (PEF) and ultrasound (US) processing

In this section, the experimental designs of PEF and US processing of milk samples are demonstrated.

#### 3.5.1 PEF processing

##### 3.5.1.1 PEF processing system

The laboratory scale PEF system used in this study was a modified version of the
OSU-4 PEF model (OSU-4, Ohio State University, Columbus, OH, USA) (Figure 3.2).

The PEF system is comprised of the following units:

i. **Twin tank system**: two product inlet tanks to feed samples into the PEF system.

ii. **Multi-jacketed heat exchanger system**: to provide an accurate temperature control of the processed samples. It consisted of a fluid handling system that could maintain a constant flow rate of the processed samples from 60 to 480 mL/min, and three independent heat exchangers which were connected to a water heating vessel.

iii. **Pulse generator**: to provide bipolar square pulses with a pulse rate from 0 to 2000 Hz while operating at voltage between 1 and 12 kV. Pulse frequency, pulse width and pulse delay time were controlled by the signal generator (Model 9310 Quantum Composers, Bozeman, Mont., USA).

iv. **Treatment chamber**: consisted of a flow-cell housing four co-field treatment chambers connected in series using stainless steel coils.

v. **Oscilloscope**: to record the applied voltage, current, pulse width and frequency using a two channel digital oscilloscope (Model TDS 2002, Tektronix Inc., Beaverton, OR, USA).

vi. **Temperature recorder**: to record temperature, two type-T thermocouples were inserted inside stainless steel housings (a total of 12 thermocouples) and connected to a 12-channel temperature recorder (ABB SM1000, Amplicon, UK). This temperature recorder was used for monitoring and recording the inlet and outlet
temperature of each treatment chamber during PEF processing. Temperature probe position 2, 4, 6, and 8 were corresponding to the inlet of treatment chamber 1, 2, 3, and 4. Temperature probe position 3, 5, 7, and 9 were corresponding to the outlet of treatment chamber 1, 2, 3, and 4 respectively. Temperature probe position 1 and 10 were corresponding to the inlet of the PEF system before any heat exchanger and outlet of the PEF system after the last heat exchanger respectively.

Figure 3.2 Schematic diagram of the laboratory-scale PEF system in current study. The temperature of the milks during the whole processing was recorded in 10 different points (T1 to T10) including the inlet and outlet of each treatment chamber.
3.5.1.2 PEF processing parameters

The geometry of the treatment chambers, parameter inputs of PEF processing, and the physical properties of milks were measured and set as shown in Table 3.2a. Using the parameters in Table 3.2a, the E-strength \((E)\), Treatment time \((t)\) and Specific energy \((E_s)\) were calculated using the following equations:

\[
E = \frac{U}{d} \tag{3.1}
\]

where \(U (= 9760 \text{ V})\) is the pulse peak voltage, \(d (= 2\text{mm})\) is the gap distance of the treatment chamber.

The total treatment time \((t)\) is given by:

\[
t = \frac{4f\tau V_t}{Q} \tag{3.2}
\]

where \(f (= 1370 \text{ Hz})\) is the input pulsing frequency, \(\tau (= 4\times10^{-6} \text{ s})\) is the pulse duration, \(V_t (= 3.5325\times10^{-9} \text{ m}^3)\) is the treatment volume, \(Q\) is the flowrate \((= 240 \text{ mL/min})\), 4 is the number of the chamber treatments.
The specific energy \( (E_s) \) is given by:

\[
E_s = \frac{4\sigma Af\tau U^2}{Qd}
\]  \hspace{1cm} 3.3

where \( \sigma \) is the electrical conductivity of milks (Table 3.2 a), \( A \) is the cross area (= \( 1.766 \times 10^6 \) m\(^2\)) of treatment chamber.

The estimated \( E, E_s \) and \( t \) of each milk are shown in black text in Table 3.3. However, the recorded pulse peak voltage \( (U) \), pulse duration \( (\tau) \) and frequency \( (f) \) by the oscilloscope during PEF processing are different from the estimated values. The recorded \( U, \tau, \) and \( f \) corresponding to the process for each milk were shown in Table 3.2 b. Thus, the actual \( E, t \) and \( E_s \) were calculated from the recorded \( U, \tau, \) and \( f \) and shown in red text in Table 3.3.
Table 3.2 (a) The geometry of the treatment chambers, parameter inputs of PEF processing, and the physical properties of milks. (b) Recorded pulse peak voltage ($U$), pulse duration ($\tau$) and frequency ($f$) by the oscilloscope during PEF processing.

### a.

<table>
<thead>
<tr>
<th>Treatment chambers</th>
<th>PEF parameter inputs</th>
<th>Milks</th>
<th>Milk conductivity (S/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Internal Diameter ($D$)</strong></td>
<td>0.0015 m</td>
<td>Pulse peak voltage ($U$)</td>
<td>9760 V</td>
</tr>
<tr>
<td><strong>Gap distance ($d$)</strong></td>
<td>0.002 m</td>
<td>Positive Pulse Duration ($\tau$)</td>
<td>0.000002 s</td>
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<tr>
<td><strong>Cross Area ($A$)</strong></td>
<td>0.000001766 m$^2$</td>
<td>Negative Pulse Duration ($\tau$)</td>
<td>0.000002 s</td>
</tr>
<tr>
<td><strong>Treatment Volume ($V_t$)</strong></td>
<td>3.5325E-09 m$^3$</td>
<td>Pulsing Frequency ($f$)</td>
<td>1370 Hz</td>
</tr>
<tr>
<td><strong>Number</strong></td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### b.

<table>
<thead>
<tr>
<th>Milks</th>
<th>Pulsing frequency (Hz) ($f$)</th>
<th>Pulse duration (s) ($\tau$)</th>
<th>Pulse peak voltage (V) ($U$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.7</td>
<td>1369.89</td>
<td>0.000003</td>
<td>10000</td>
</tr>
<tr>
<td>pH 7.1</td>
<td>1369.89</td>
<td>0.000003</td>
<td>10200</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>1369.89</td>
<td>0.000003</td>
<td>10000</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>1369.89</td>
<td>0.000003</td>
<td>10000</td>
</tr>
<tr>
<td>0 mM EDTA</td>
<td>1369.89</td>
<td>0.000003</td>
<td>9900</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>1369.89</td>
<td>0.000003</td>
<td>9500</td>
</tr>
<tr>
<td>20 mM EDTA</td>
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<td>0.000003</td>
<td>8300</td>
</tr>
<tr>
<td>30 mM EDTA</td>
<td>1369.89</td>
<td>0.000003</td>
<td>8200</td>
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</table>
Table 3.3 Estimated (in black text) and actual (in red text) key parameter outputs of PEF processing.

<table>
<thead>
<tr>
<th>Milks</th>
<th>E-strength (kV/cm) ($U$)</th>
<th>Treatment time (µs) ($t$)</th>
<th>Specific Energy (kJ/L) ($E_s$)</th>
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<tr>
<td>pH 6.7</td>
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<td>50.0</td>
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<td>457.30</td>
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<td>19.36</td>
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<td></td>
<td>51.0</td>
<td>29.03</td>
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</tr>
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<td>pH 7.5</td>
<td>48.8</td>
<td>19.36</td>
<td>295.04</td>
</tr>
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<td>50.0</td>
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<td>464.56</td>
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<td>19.36</td>
<td>295.04</td>
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<td>19.36</td>
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<tr>
<td></td>
<td>49.5</td>
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<td>433.97</td>
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<td></td>
<td>41.0</td>
<td>29.03</td>
<td>532.00</td>
</tr>
</tbody>
</table>

3.5.1.3 PEF processing of milks

Before PEF processing milks were adjusted to pH 6.7, 7.1, 7.5, and 8.0, or added with EDTA at various concentrations (0, 10, 20 and 30 mM) at pH 6.7. All milks were equilibrated to 20 ºC using a water bath. Milks were either circulated through the PEF unit with the electric field using the conditions specified in Table 3.2 (PEF-treated milks) or circulated through the PEF unit with electric field turned off (non-PEF treated milks). This experiment was carried out to assess the effect of heat and shear experienced by the sample in the PEF unit. All treatments, both with the electric field applied or not applied, were conducted in duplicates on at least two different
occasions. In addition to these samples, unprocessed samples which represent the starting milks were characterised (control milks).

Non-PEF treated milks were circulated through the PEF system without pulsing whilst being exposed to time-temperature profiles to mimic as far as possible the ohmic heat and the shear effects experienced during PEF processing. This was achieved by manipulating the heat exchangers, where the temperatures were set to 45 °C, 72 °C and 20 °C corresponding to the first, second and third heat exchangers to simulate the ohmic heating during the PEF processing.

3.5.2 US processing

3.5.2.1 US processing reactors

Various US processing units with different processing frequency were used: a 20 kHz horn-transducer unit (Branson Digital Sonifier, Branson Ultrasonic Corporation, CT, USA), a 20 kHz horn-transducer unit (UPI2000hd, Hielscher Ultrasonics GmbH, Warthestr. 21, 14513 Teltow, Germany), a 400 kHz plate-transducer unit (Submersible Transducers, Sonosys Ultraschallsysteme GmbH, Neuenburg, Germany) and a 1600 kHz plate-transducer unit (Nebulizer, APC International Inc. Mackayville, PA, USA). As these transducers had different dimensions, different sized metal treatment chambers were built to hold the processed samples in which the transducers were completely immersed during the US processing. The transducer active area/volume of
sample ratio was maximised to reach a maximum specific energy input within each device. The dimensions of the transducers and corresponding treatment chambers are shown in Figure 3.3.

Figure 3.3 Dimensions of transducers and treatment chambers. (a) 20 kHz horn-type US processing unit (Branson Digital Sonifier). (b) 20 kHz horn-type US processing unit (Hielscher). (c) 400 kHz plate-type US processing unit (Sonosys). (d) 1600 kHz plate-type US processing unit (Nebulizer).
3.5.2.2 Measurement of power input in US processing

The power input \((P)\) of each US processing unit in its treatment chamber was characterized by calorimetry. In order to carry out the calorimetric study the system was insulated to ensure no heat loss in a period of time (120 s). The increase of the sample temperature in foam insulated treatment chambers, induced by US processing was measured. The power input was calculated using equation:

\[
P = m \cdot C_p \cdot \frac{dT}{dt}
\]

where \(P\) is power input in watts (W), \(C_p = 4.18 \text{ J} / \degree\text{C} / \text{g}\) is the specific heat capacity of water, \(m\) is the mass of sample (g), and \(dT/dt\) is the sample temperature increase rate in a defined processing time of 120 s. At 100 % power level, the power inputs of the 20 kHz US unit (Branson), 20 kHz US unit (Hielscher), the 400 kHz US unit, and the 1600 kHz US unit were 31.79 W, 51.90 W, 85.69 W, and 3.53 W, respectively.

3.5.2.3 Determination and selection of the specific energy input

In this study, the ultrasonic processing conditions were expressed by the specific energy input \((E_s)\), which is defined as the energy input per mass of the material being processed. \(E_s\) is given by:

\[
E_s = \frac{P_t}{m}
\]
where $E_s$ is specific energy (kJ/kg), $P$ is power input (W), $t$ is the processing time (s) and $m$ is the mass of sample (g). In order to separate the effects of energy and ultrasound frequency, a constant value of $E_s$ was selected across all US units at their respective frequencies. The $E_s$ was chosen based on a preliminary evaluation of particle size in milk after ultrasonic treatment at 20 kHz at various specific energy inputs.

Reconstituted skim milk (10 % TS) adjusted to pH 6.7 was treated in the Branson Digital Sonifier at 20 kHz and six specific energy inputs within the range of 0 – 572 kJ/kg. Casein micelle size was measured using the methodology described in Section 3.2.1.1. As shown in Figure 3.4, the mean particle size of the non-sonicated milk was 202 nm at pH 6.7. The ultrasonication of milk (pH 6.7) at 20 kHz decreased the particle size of the casein micelles. With increasing specific energy input from 0 to 286 kJ/kg, there was a steady decrease in casein micelle size to 183 nm and there was no further decrease in particle size when the specific energy input was increased to 572 kJ/kg. The result showed that ultrasound processing at $E_s = 286$ kJ/kg resulted in casein particle disruption to a large extent in milk.

The application of the same $E_s$ (286 kJ/kg) to samples in all other US processing units was achieved by varying the sample volume and the processing time as shown in Table 3.4.
Figure 3.4 Average casein micelle size in the 20 kHz-Us treated milk at pH 6.7 at increasing processing specific energy inputs

Experiments in Chapter 5 are based trials with the 20 kHz (Branson), 400 kHz and 1600 kHz units while the 20 kHz (Hielscher) unit was used for Chapter 6. The 20 kHz (Hielscher) unit became available at a later stage of this study and can achieve higher specific energy compared to the 20 kHz (Branson) unit.
Table 3.4 Processing sample volume and time in different US processing units to achieve specific energy 286 kJ/kg.

<table>
<thead>
<tr>
<th>US processing unit</th>
<th>Sample volume (mL)</th>
<th>Processing time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 kHz (Branson)</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>20 kHz (Hielscher)</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>400 kHz</td>
<td>300</td>
<td>17</td>
</tr>
<tr>
<td>1600 kHz</td>
<td>25</td>
<td>34</td>
</tr>
</tbody>
</table>

3.5.2.4 Sample temperature during US processing

The sample temperature during US processing was measured using a thermocouple placed in the centre of treatment container. As shown in the temperature profiles obtained during the application of ultrasound (Figure 3.5), the temperature did not rise above 30°C during processing. Therefore, the sample remained below the temperature threshold for heat-induced denaturation of whey proteins of ~ 70 °C (Oldfield, Singh, Taylor & Pearce, 1998). In addition, ultrasonic processing at this bulk temperature allowed separation of bulk heat- and ultrasound-induced effects on proteins.
Figure 3.5 Temperature profile in the sample during ultrasonication at 20 kHz (Branson) (Black line), 20 kHz (Hielscher) (Grey line), 300 kHz (Red line) and 1600 kHz (Green line).

### 3.5.2.5 Characterisation of the chemical effects in US processing

A methodology to follow the generation of free radicals resulting from ultrasonic cavitation was developed by Ashokkumar et al. (2008). It is based on the quantification of the amount of \( \text{OH}^* \) generated. During US processing of a liquid media, generation of free radicals follows the mechanism as shown in reactions 2.1 \( (\text{H}_2\text{O} \rightarrow \text{OH}^* + \text{H}^*) \) and 2.3 \( (\text{OH}^* + \text{OH}^* \rightarrow \text{H}_2\text{O}_2) \) in Chapter 2.
The method is based on the quantification of $\text{H}_2\text{O}_2$ (Reaction 2.3) by a colorimetric assay utilizing Iodine as shown in reaction 3.1 and 3.2.

\[
\text{H}_2\text{O}_2 + 2\Gamma \rightarrow \text{I}_2 + 2\text{OH}^- \quad 3.1
\]
\[
\text{I}_2 + \Gamma \rightarrow \text{I}_3^- \quad 3.2
\]

$\text{H}_2\text{O}_2$ can oxidize iodide ions ($\Gamma$) and generate molecular iodine ($\text{I}_2$) (reaction 3.1). When the $\Gamma$ is in excess in the medium, $\text{I}_3^-$ complex will be formed (reaction 3.2), which has an absorption band at $\lambda$ 352 nm. The concentration of $\text{OH}^*$ is then able to be calculated using the absorption and molar extinction coefficient.

In this study, MilliQ water was subjected to US processing (20 kHz (Branson), 400 kHz and 1600 kHz) at different specific energy inputs (0 - 430 kJ/kg). US-treated solutions were then immediately mixed with 1 mL of freshly prepared iodide reagent (0.4 M KI, 0.05 M NaOH, $1.6 \times 10^{-4}$ M $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}.4\text{H}_2\text{O}$) and 1 mL of 0.1 M KHC$_8$H$_4$O$_4$. Mixed solution was then transferred to a cuvette to measure the absorbance at 352 nm which is corresponding to the absorption of $\text{I}_3^-$, in a spectrophotometer (SHIMADZO, UV-1700 Pharma Spec). The values of the absorbance were then converted to the concentration of $\text{H}_2\text{O}_2$ as following:

\[
C = \frac{3A}{\varepsilon} \quad 3.6
\]
Where $C$ [M] is the concentration of $H_2O_2$, $A$ is the absorbance at 352 nm, and $\varepsilon$ [26,400 M$^{-1}$ cm$^{-1}$] is the molar extinction coefficient.

The concentration of OH$^*$ yields is calculated and are present as a function of the specific energy input of the US processing in Figure 3.6. Higher concentration of OH$^*$ formed during US processing indicates a higher extent of chemical effect from the ultrasonication. The concentration of OH$^*$ increased rapidly with the increase of specific energy input at 400 kHz US processing. The increasing trend was also observed in 20 kHz and 1600 kHz processing except the increase was much slower compared to that in 400 kHz processing. It suggests 400 kHz US processing had the largest extent of chemical effects among these 3 frequencies of US processing.

![Figure 3.6 Concentration of OH radicals generated in MilliQ water upon ultrasonication at frequency 20 kHz (■), 400 kHz (●), and 1600 kHz (▲).](image-url)
3.6 Statistics

All treatments and measurements were performed at least in duplicate. Statistical analysis of the data was performed using SPSS 17.0 (SPSS Inc. 2008). A confidence level of 0.05 was used to evaluate significant differences.
Chapter 4

Pulsed electric fields processing effects on reconstituted skim milk

Summary

Reconstituted skim milks (10 % TS) at natural pH 6.7, or modified by pH adjustment in the range of pH 6.7 to 8.0, or modified by the addition of EDTA (up to 30 mM) at constant pH 6.7 were subjected to PEF processing. Except for milks at pH ≥ 7.5, PEF treatment did not affect size or ζ-potential of the milk particles, the amounts of protein in the serum or the state of the whey proteins, and the distribution of phosphate between the serum and the micellar phase. For milks at pH ≥ 7.5, a decrease in the size of the milk particles and an increase in the amount of proteins in the serum were obtained. SDS-PAGE measurements showed that the amount of caseins increased in the serum phase, while the whey proteins were not affected. The decrease in the size of the milk proteins was attributed to the dissociation of the casein micelles. This study also demonstrated that the effects on milks at pH ≥ 7.5 subjected to PEF processing were due to ohmic heating during the PEF treatment, and not due to the PEF treatment per se.
4.1 Introduction

Most research on PEF effects on milk-based products has focused on microbial and enzymatic inactivation (Bendicho, Barbosa-Cánovas, & Martin, 2002; Evrendilek, Zhang, & Richter, 2004; Martín-Belloso et al., 1997; Martín, Qin, Chang, Barbosa-Cánovas, & Swanson, 1997; Picart, Dumay, & Cheftel, 2002; Smith, Mittal, & Griffiths, 2002). There have been limited reports on the PEF effects on milk proteins and the results obtained from the literature are inconclusive (Barsotti, Dumay, Mu, Diaz, & Cheftel, 2002; Floury et al., 2006; Hemar et al., 2011; Perez & Pilosof, 2004; Xiang, 2007; Yu et al., 2009 & 2012). Both the PEF conditions and the partitioning of the proteins in milk can potentially influence the processing-induced effects obtained when milk is subjected to PEF processing. In this study, the effects of PEF processing on milks in which the state of association of the caseins was altered by alkaline pH or by the addition of a calcium chelating agent (EDTA) were examined. In order to separate the effects induced by the ohmic heating, which occurs during the PEF treatment, from the electromechanical stress on proteins due to the electric field applied during PEF treatment, the temperature profile of the milks during PEF processing was measured. Selected milks (milks with pH adjustment) were then exposed to a heat treatment alone that closely mimicked the ohmic heating profiles during PEF processing. The properties of milks examined were particle size and ζ-potential by dynamic light scattering (DLS), the amount of protein and type of caseins in milk serum by nitrogen analysis and SDS-PAGE, and the distribution of phosphorus between the serum and micellar phases of milk using 31P NMR
4.2 Experimental methods

4.2.1 Milk samples

Reconstituted skim milks (10% TS) at pH 6.7, 7.1, 7.5, and 8.0 or with EDTA addition 0, 10, 20, and 30 mM at constant pH 6.7 were prepared as described in Section 3.1.2.

4.2.2 PEF processing

Milks were subjected to PEF treatment as described in Section 3.5.1.3. The parameters of the PEF processing including E-strength, total treatment time and specific energy were listed in Section 3.5.1.2, Table 3.3. The temperature of the milks at different positions in the system during processing was recorded. The temperature probes are shown in Chapter 3, Section 3.5.1.1, Figure 3.2.

Milks that were put through the PEF unit with application of the electric field will be named “PEF-treated milks”. Milks that were circulated through the PEF unit with electric field turned off will be named “non-PEF treated milks”. Milks that were unprocessed will be named “control milks”.
The particle size in milks and the $\zeta$-potential of the particles in milks were measured by dynamic light scattering using a Malvern zetasizer as described in Section 3.2.1.1, except for that the milks were diluted in deionised water instead of ultrafiltrate in the $\zeta$-potential measurements.

Milk serum was obtained by centrifugation as described in Section 3.2.2.1. The total nitrogen content in milk serum was measured using a Leco FP2000 as described in Section 3.2.2.2 and electrophoresis under reducing conditions was performed as described in Section 3.2.2.3.

The phosphoseryl residues (SerP) and inorganic phosphate (Pi) in milk were examined by $^{31}$P NMR as described in Section 3.2.2.4.

**4.3 Results**

**4.3.1 Temperature increase in milks during PEF treatment**

During PEF treatment the temperature of the sample increases due to ohmic heating, despite implementation of a cooling system using heat exchangers placed after each PEF chamber. The temperatures measured at different positions of the PEF system are
reported in Figure 4.1. The passage of the milks successively in 4 PEF chambers raised the temperature from \(\sim 20\ ^\circ C\) (initial sample temperature) to \(\sim 70\ ^\circ C\) (temperature at the outlet of the 4th PEF chamber). Then processed milks were immediately cooled down to \(\sim 25\ ^\circ C\). Temperature probe positions 2, 4, 6, and 8 corresponded to the inlet of treatment chamber 1, 2, 3, and 4, respectively. Temperature probe positions 3, 5, 7, and 9 corresponded to the outlet of treatment chamber 1, 2, 3, and 4 respectively (see Chapter 3, Section 3.5.1.1, Figure 3.2). The PEF pulsing in each treatment increased the temperature of the milk by \(\sim 25 – 30\ ^\circ C\). Therefore the sample temperature at the outlet of the 4th treatment chamber reached \(\sim 70\ ^\circ C\) despite the cooling after each treatment chamber. The temperature profiles of milks at different pH (solid symbols) or with different levels of added EDTA (open symbols) were nearly identical. The temperature profile of the milk circulating through the PEF system without applying electric fields is indicated in dotted line and showed a similar increasing trend to that of the milks during PEF treatment. Solid lines and dashed lines are the calculated temperatures for milk at pH 6.7 (without EDTA addition) and milk with 30 mM added EDTA respectively, which will be discussed in Section 4.4.1.
Figure 4.1 Temperature at different locations of the PEF unit for different milk. Milks at pH 6.7 (□), pH 7.0 (●), pH 7.1 (▲) or pH 8.0 (▼); or milks (pH 6.7) with 0 mM (□), 10 mM (○), 20 mM (△) or 30 mM EDTA (▽). Temperatures were measured at: 1. Sample temperature prior to PEF treatment; 2. Inlet 1st PEF chamber; 3. Outlet 1st PEF chamber; 4. Inlet 2nd PEF chamber; 5. Outlet 2nd PEF chamber; 6. Inlet 3rd PEF chamber; 7. Outlet 3rd PEF chamber; 8. Inlet 4th PEF chamber; 9. Outlet 5th chamber; 10. Sample temperature after last heat exchanger. Solid and dashed lines are calculated temperatures for milk (pH 6.7 without added EDTA) and milk with 30 mM added EDTA. Dotted line corresponds to temperature profile used on milk sample (pH 6.7) with the PEF unit switched off.
4.3.2 Particle size and $\zeta$-potential

Particle size measurements of the milks at different pHs and different levels of added EDTA are presented in Figure 4.2. The particle size measurements showed that the control milks, PEF-treated and non-PEF treated milks with no added EDTA contained particles exhibiting a monomodal distribution with sizes ranging from ~90 nm to 700 nm (Figure 4.2 a) over the pH range 6.7-8.0. The average particle diameters in control and PEF-treated milks are reported in Figure 4.2 c. When milk pH was increased from 6.7 to 8.0, the average particle size in control milks increased from 207 nm to 223 nm. The same trend was observed in PEF-treated milks and non-PEF treated milks at pH 6.7 to 7.5. However, at pH 8.0, the particle size of both PEF-treated and non-PEF treated milks decreased to 200 nm.

In the case of control and PEF-treated milks with added EDTA up to 20 mM, the particle size distribution is monomodal with sizes ranging from 80 nm to 600 nm (Figure 4.2 b). At 30 mM added EDTA, the particle size distribution, although monomodal, was made of particles having a diameter in the range of 10 to 400 nm. This decrease in particle size in milks with 30 mM EDTA addition is also apparent in Figure 4.2 c (open symbols). This shift toward small sizes at 30 mM EDTA is an indication of the dissociation of the casein micelle. The average particle size of milks at all levels of EDTA addition was not significantly different ($p > 0.05$) for both control and PEF treated milks.
Figure 4.2 Particle size distribution of control (solid symbols), PEF-treated (open symbols) or non-PEF treated (pattern symbols) for (a) milks at pH 6.7 (■, □, △), 7.1 (●, ○, △), 7.5 (▲, △, △) or 8.0 (▼, ▼, △) and (b) milks with 0 mM (■, □), 10 mM (●, ○), 20 mM (▲, △) or 30 mM (▼, ▼) added EDTA. (c) Reports the calculated intensity average diameter (z-average) as a function of pH (solid symbols) or EDTA concentration (open symbols). In (c) control milks (■, □); PEF-treated milks (●, ○); and non-PEF treated milks (▲) in the PEF unit using the temperature profile shown in Figure 4.1 (dashed line). Error bars represent standard deviation of duplicates.

ζ-Potential is an indication of the surface charge of particles. The smaller the absolute value of the ζ-potential is, the more likely that the particles tend to aggregate. In Figure 4.3, the ζ-potential measurements of control, PEF-treated and non-PEF treated milks are reported. The ζ-potential values of the milks decreased slightly with the
increase in pH, from \(-25\,\text{mV}\) at pH 6.7 to \(-27.5\,\text{mV}\) at pH 8.0, which may be due to the increase in negative charges on the casein with the increase in pH. In the case of EDTA, the \(\zeta\)-potential values decrease from \(-25\,\text{mV}\) without EDTA addition to \(-37.5\,\text{mV}\) with 30 mM EDTA addition at natural milk pH 6.7. As expected the value of the \(\zeta\)-potential decreases with the increase in pH, as the milk proteins become more negatively charged. However, at a given pH or EDTA concentration, there is no significant difference (p > 0.05) in the \(\zeta\)-potential of the control, PEF treated and non-PEF treated milks.

![Graph showing \(\zeta\)-potential of control (■, □), PEF treated (●, ○), and non-PEF treated milks (▲) as a function of pH (solid symbols) or added EDTA (open symbols). Error bars represent standard deviation of duplicates.](image)

Figure 4.3 \(\zeta\)-potential of control (■, □), PEF treated (●, ○), and non-PEF treated milks (▲) as a function of pH (solid symbols) or added EDTA (open symbols). Error bars represent standard deviation of duplicates.
4.3.3 Proteins in the serum phase

The total protein content in the serum (expressed as the percentage of the total proteins in milk) of different milks was measured and is shown in Figure 4.4. In control milks at different pH or added EDTA, the protein content in the serum increased with the increase in pH or added EDTA level. This was due to the partial dissociation of micellar caseins into the serum at higher milk pH or when EDTA sequestrated Ca. For milks at natural pH 6.7 and at pH 7.1, and for EDTA-treated milks, there was no significant difference (p > 0.05) in the amount of protein in the serum phase for control, PEF-treated and non-PEF treated milks. However, at pH ≥ 7.5, both the PEF-treated and non-PEF treated milks showed an increase in the amount of protein in the serum, compared to control milks at corresponding pH.
Figure 4.4 Amount of serum proteins of control (■, □), PEF-treated milks (●, ○) and non-PEF treated (▲) as a function of pH (solid symbols) or added EDTA (open symbols). Error bars represent standard deviation of duplicates.

SDS-PAGE measurements under reducing conditions were carried out to show the type and amount of individual protein in the milk serum. α-, β-, κ-Caseins, β-Lg and α-Lac in the serum phase were quantified and shown in Figure 4.5 c (reducing gels are shown in Figure 4.5 a). It is noted that the κ-caseins in sera of milks at pH ≤ 7.1 could not be quantified due to the low level of κ-casein present in the serum phase. In the case of milks at different pH, the α-, β- and κ-casein content in the serum phase increased with the increase of the pH in control, PEF-treated and non-PEF treated
milks. At pH 6.7 and 7.1, neither treatment (PEF and non-PEF) changed the casein content in the serum, compared to that of control milks. However, in the case of milks with a pH ≥ 7.5, both the PEF-treated and the non-PEF treated milks showed an increase in the amount of the caseins (α-casein, β-casein and κ-casein) in the serum compared to the control milks. On the other hand, regardless of pH, the levels of both whey proteins remained unchanged by PEF and non-PEF treatments. All these observations are consistent with the increase in total serum proteins being due to partial dissociation of micellar caseins.

In the case of milks with added EDTA, casein (α-casein, β-casein and κ-casein) content in the serum increased with the increased EDTA addition, whereas the content of whey proteins did not change regardless of the EDTA addition levels. Moreover, neither PEF treatment nor non-PEF treatment affected the casein or whey protein content in the serum phase in any EDTA added milks.

In order to determine the PEF effects on the aggregation of whey proteins, SDS-PAGE was also carried out in non-reducing conditions as shown in Figure 4.5 b. There were some aggregates observed on the wells of the non-reducing gels in both milks at various pH and milks at different EDTA addition levels. However, some aggregates were already present in milks before PEF treatment. This small amount of aggregate was due to the original milk powder production. PEF treatment did not change the amount of these aggregates. These results indicated that PEF treatment did
not induce whey protein aggregation.
Figure 4.5 SDS-PAGE (a) reducing gels of sera of milks at various pH (left) or with various level of EDTA addition (right). Ctl: Control milk serum; PEF: PEF-treated milk serum; Non PEF: non-PEF treated milk serum; um: untreated milk. (b) non-reducing gels of sera of milks at various pH (left) or with various level of EDTA addition (right). (c) reducing gels quantification of serum proteins as a function of pH (left) or added EDTA (right) for control milks (■,□); PEF-treated milks (●,○); or non-PEF treated milks (▲). Error bars represent standard deviation of duplicates.
4.3.4 Phosphate distribution in milk

A typical $^{31}$P NMR spectrum of milk at pH 6.7 (no EDTA addition) is shown in Figure 4.6 a. This spectrum is comparable to that presented in the study of Belton et al. (1985). The major peak at chemical shift 2.2 ppm represents inorganic phosphate (Pi). The peaks at chemical shifts between 2.9 – 4.8 ppm correspond to phosphoseryl residues (SerP). The areas under the peaks in a $^{31}$P NMR spectrum are proportional to the concentrations of the different species of phosphorus in the sample. These two regions were integrated and quantified as shown in Figures 4.6 a and b. In control milks, the change in phosphate (Pi) concentration with pH was very small although the SerP concentration increased slightly with increase of pH. Regardless of pH, PEF treatment did not affect the Pi and SerP concentration in milks. In control milks with EDTA additions, both the Pi and SerP concentrations increased markedly with the increase in EDTA addition level. However, PEF treatment had no significant effect (p > 0.05) on the Pi and SerP concentrations compared to concentrations in control milks with corresponding EDTA additions.
Figure 4.6 $^{31}$P NMR Analysis (a) Spectrum of milk at pH 6.7 (without EDTA). (b) Peak area of inorganic phosphate (Pi) in milks at different pH (solid symbols), or at different levels of added EDTA (open symbols). (c) Peak area of phosphoseryl residues (SerP) in milks at different pH (solid symbols), or at different levels of added EDTA (open symbols). Symbols are: Control milks (■, □) and PEF-treated milks (●, ○). Error bars represent standard deviation of duplicates.
4.4 Discussion

4.4.1 Ohmic heating of samples during PEF processing

Although PEF processing is considered to be a non-thermal processing technology, ohmic heating in processed samples during treatment is inevitable due to the conversion of electrical energy into heat. There have been a few studies investigating the electrically induced heat effects on microbial and enzyme inactivation in PEF processing (Guerrero-Beltran, Sepulveda, Gongora-Nieto, Swanson, & Barbosa-Cánovas, 2010; Jaeger, Meneses, Moritz, & Knorr, 2010) and the optimization of PEF treatment chamber designs by modelling the temperature distribution in the treatment chambers (Fiala, Wouters, Van Den Bosch, & Creyghton, 2001; Lindgren, Aronsson, Galt, & Ohlsson, 2002). Besides the geometry and configuration of the treatment chamber, the sample itself can have impact in the ohmic heating during treatment. Theoretically, for PEF treatment, the increase in temperature $\Delta T$ is given by (Lindgren, Aronsson, Galt & Ohlson, 2002):

$$\Delta T = \frac{\sigma d \tau f}{2 \rho C_v u_{avg}} E^2$$  \hspace{1cm} 4.1

where $\sigma$ is the electrical conductivity, $d$ (=2 mm) is the PEF chamber gap, $\tau$ (= $4 \times 10^{-6}$ s) is the pulse duration, $\rho$ (=1031 kg/m$^3$) the milk density, $C_v$ (= 4200) its heat capacity.
The fluid velocity \( u_{avg} \) in the PEF chamber is given by:

\[
    u_{avg} = \frac{Q}{\pi R^2} \quad 4.2
\]

where \( Q (= 4\times10^{-6} \text{ m}^3/\text{s}) \) is the flow rate of the milk samples and \( R (= 0.75 \text{ mm}) \) the radius of the PEF chamber. Because the increase in temperature \( \Delta T \) is proportional to the electrical conductivity \( \sigma \) (Eq. 4.1), measurements were carried out to determine the value of \( \sigma \) for different milk samples, and the results are reported in Figure 4.7. The electrical conductivity increases with the increase in pH or with the increase in added EDTA. This is expected since the addition of NaOH or EDTA increased the levels of conductive materials in the samples. In addition, the increase in pH or added EDTA leads to the dissociation of the casein micelles and the release of cations and anions into solution. In the case of high pH, the dissociation of the casein micelles is due to the repulsive electrostatic forces between the casein molecules (Ahmad, Piot, Rousseau, Grongnet, & Gaucheron, 2009; Liu & Guo, 2008).
Figure 4.7 Conductivity of milks at different pH (■), or at different levels of added EDTA (□). Error bars represent standard deviation of duplicates.

In the case of EDTA, the sequestration of Ca leads to the dissociation of the micellar calcium phosphate (MCP) which has a role in the cross-linking of the caseins (Udabage, Mckinnon, & Augustin, 2000). $^{31}$P NMR measurements have shown that the amount of Pi increased markedly in the case of milks with added EDTA (Figure 4.6 b); whereas, in the case of milks at higher pH, no increase in Pi was observed by $^{31}$P NMR. Using Eq. 4.1 and the values of the conductivity reported in Figure 4.7, the theoretical increase in temperature $\Delta T$ due to ohmic heating during PEF treatment can
be calculated. In Figure 4.1, the expected temperature $T$, after passing through each PEF chamber, is reported for milk (pH 6.7 without EDTA) (solid line) and milk with 30 mM EDTA (dashed lines). The temperature $T$ is calculated as:

$$T = T_0 + is \quad 4.3$$

where $T_0$ is the temperature of the sample before entering the PEF chamber. For example $T_0 = 20 \, ^\circ C$ (room temperature) before entering the first PEF chamber. Eq. 4.1 gives $\Delta T = 19.8 \, ^\circ C$ for milk (pH 6.7 without EDTA) and $\Delta T = 32 \, ^\circ C$ for milk with added EDTA. The calculated temperature of milk (pH 6.7 without EDTA) (solid lines) at the inlet of treatment chamber 2, 3 and 4 was ~ 5 to 15 °C lower than the recorded temperature. This may result from the heterogeneity of the temperature distribution in the treatment chamber causing local hot spots and leading to the higher sample temperature (Fiala, Wouters, Van Den Bosch, & Creyghton, 2001). On the other hand, the calculated temperature of milk with 30 mM EDTA (dashed lines) was higher than the recorded temperature at the inlets of all treatment chambers. This may be due to the variation of the applied E-strength during PEF processing which is shown in Table 3.3 in Section 3.5.1.2, Chapter 3. The theoretical E-strength in milk with 30 mM EDTA was 48.8 kV/cm, whereas the actual E-strength in this milk was 41.0 kV/cm. Hence the recorded temperature increase during PEF processing was lower than the calculated ones according to Eq. 4.1.
Although the high temperature (∼70 °C in Figure 4.1) experienced by the milks was for a very short time (residence time in one PEF chamber is ∼9 ms and total residence time in PEF unit is less than 55 sec), its effect on the milk protein cannot be neglected. In particular, whey proteins are well known to denature and aggregate under heat-treatment (Oldfield et al., 1998).

### 4.4.2 Reduction of particle size in milks at pH 8.0 during PEF treatment

As shown in Figure 4.2 c, the average diameter of the particles was found to be ∼200 nm in control milks at natural pH (6.7). This is in agreement with the value previously reported for the casein micelles in milk (Anema, 2007; Holt, 1975; Wade, Beattie, Rowlands, & Augustin, 1996). When the pH of the milk was increased, the average diameter increased to reach a value of ∼220 nm. This increase in diameter can be attributed to the swelling of the casein micelle as a result of increasing electrostatic repulsions between the proteins (Liu & Guo, 2008). PEF treatment did not change the particle size in milks at pH up to 7.5. But at pH 8.0, the average diameter of the particles decreased by ∼20 nm after PEF treatment. To determine the effect of the temperature on the milk proteins, milks were also circulated through the PEF unit without PEF treatment (non-PEF treated milks), but with the heat exchangers set at different temperatures to mimic the ohmic heating effects during PEF treatment. Particle size measurements of non-PEF treated milk at pH 8.0 also showed a ∼20 nm
reduction in the average diameter of milk particles compared to that of control milk at pH 8.0. This demonstrates clearly that the reduction of the size of the casein micelle at high pH during PEF treatment was due to the ohmic heat generated during the PEF and was not of electrical origin.

4.4.3 Increase in protein content in serum of milks at pH 8.0 by PEF treatment

The increase in total protein content in serum of milks at pH 8.0 after PEF treatment was shown in Figure 4.4. A similar increase in total protein content in serum was also observed in non-PEF treated milk at pH 8.0. These results support the theory that the reduction in particle size in milks at pH 8.0 after treatments was due to the dissociation of micellar caseins. Moreover, SDS-PAGE measurements under reducing conditions showed an increase of caseins in milks at pH 8.0 after PEF treatment and after circulation through the PEF system without applying electric fields (Figure 4.5a). These results indicated that reduction in particle size in milks after PEF-treatment was due to the heating-induced dissociation of casein micelles. It is not surprising that the heat-induced micelle disruption only happened in milks at high pH. Numerous studies have shown that alkaline pH in milk promotes heat-induced micelle dissociation (Anema & Klostermeyer, 1997a & 1997b; Kudo, 1980; Nieuwenhuijse, Vanboekel, & Walstra, 1991).
The denaturation temperature (~ 70 °C) of whey protein was reached during the PEF treatment. In addition, higher pH is known to increase the heat-induced aggregation rate of β-Lg (Hoffmann & Van Mil, 1997). However, the content of whey proteins in milk at pH 8.0 was unchanged after PEF treatment as shown in Figure 4.6 a. Aggregation of whey proteins in milk at pH 8.0 after PEF treatment was not observed in SDS-PAGE gels in non-reducing condition as shown in Figure 4.6 b. This is in agreement with Sui et al. (2011) who showed that PEF (35 kV/cm, at 30 – 75 °C, 19.2 µs) treatment did not affect the physicochemical characteristics of 1% whey protein isolates. It is likely that, although temperatures as high as 70 °C were reached during PEF treatment, the samples experienced these high temperatures only for a short time and, given the lower concentrations of the whey proteins, aggregation and gelation did not occur.

4.5 Conclusion

This study investigated the effects of PEF treatment on the physical and chemical state of the casein micelles in milk. The current published literature does not provide a clear picture of the effects of PEF on milk. This is partly because many different PEF conditions are used in various studies, which makes the comparison between studies difficult. In this study, the PEF unit was operated to its maximum E-strength of ~ 50 kV/cm. In addition, the casein micelle was also weakened by either increasing the pH
of the milk or by the addition of EDTA, which is known to lead to the dissociation of the casein micelles. Despite these extreme conditions, it was found that in all milks investigated, PEF treatment affected only the milk at the highest pH considered (pH 8.0). These milks showed a smaller particle size than the control milks at the same pH. The decrease in the particle size is due to the dissociation of the casein micelles, as shown by the measurements of the amount of protein present in the serum; and SDS-PAGE identified these as being caseins. However, this effect of PEF treatment on the particle size of milk at pH 8.0 is due to ohmic heating which was confirmed by circulating the reconstituted milk sample at pH 8.0 in the PEF unit, with the PEF treatment switched off, while imposing a heating profile (similar to that generated during PEF) using heat exchangers.
Chapter 5

Ultrasound processing effects on reconstituted skim milk

Summary

Reconstituted skim milks (10 % w/w total solids, pH 6.7 – 8.0) were ultrasonicated (20, 400 or 1600 kHz at a specific energy input of 286 kJ/kg) at a bulk milk temperature of < 30 °C. Ultrasound application to milk at different pH altered the properties of the milk, with greater effects at higher pH and lower frequency. Ultrasound disrupted casein micelles causing release of protein from the micellar to the serum phase, without affecting the partitioning of phosphate in milk. The released protein re-associated to form aggregates of smaller size with similar surface charge to the casein micelles in the original milk. Ultrasound may thus be used as a physical intervention to alter the partitioning of caseins between the micellar and serum phases in milk. The altered protein equilibria induced by ultrasound treatment may have potential for the development of milk with novel functionality.
5.1 Introduction

Most studies in dairy systems have involved the use of low frequency ultrasound in the range of ~ 20 to 100 kHz. For example, application of low frequency ultrasound (20 kHz) to whey protein concentrates, milk protein retentates and calcium caseinate solutions reduced particle size and viscosity (Zisu et al., 2010). Gordon and Pilosof (2010) found that low frequency (20 kHz) US at high intensity could be used to control the size of whey protein particles where either disruption or flocculation of protein particles could be obtained by varying the conditions of ultrasound application. Experiments on re-assembled casein micelles confirmed that ultrasound (35 kHz, 130 kHz or dual-frequency of 24 kHz and 130 kHz) disrupted casein micelles and re-formed smaller particles (Madadlou et al., 2009a, 2009b & 2012).

In this study, reconstituted skim milk at pH 6.7 – 8.0 was ultrasonicated at 20, 400 or 1600 kHz at the same specific energy input (286 kJ/kg) in order to isolate the effects of frequency from those of power. The processing temperature of the bulk milk was maintained at < 30 °C which is below the temperature (~ 70 °C) for whey protein denaturation (Oldfield et al., 1998). The consequences of ultrasound treatment on the particle size and ζ-potential of protein particles, the integrity of the casein micelles, the distribution of proteins between the serum and micellar phases of milk as well as the nature and composition of soluble aggregates in the serum were determined.
5.2 Experimental methods

Reconstituted skim milks (10 % TS) at pH 6.7, 7.1, 7.5, and 8.0 were prepared as described in Section 3.1.2. Milks were then subjected to ultrasound processing at 20 kHz (Branson system), 400 kHz, or 1600 kHz at the same specific energy input of 286 kJ/kg as described in Section 3.5.2. The bulk temperature of the milk during US processing was controlled < 30 °C as described in Section 3.5.2.4, Figure 3.5.

After processing, the particle size in milks and the \( \zeta \)-potential of the particles in milks were measured by dynamic light scattering using a Malvern zetasizer as described in Section 3.2.1.1. Milk serum was obtained as described in Section 3.2.2.1 and the following analyses were carried out in milk serum: the total nitrogen content in milk serum was measured as described in Section 3.2.2.2; electrophoresis under reducing conditions was performed as described in Section 3.2.2.3; the phosphoseryl residues (SerP) and inorganic phosphorus (Pi) in milk serum were examined by \( ^{31} \text{P} \) NMR as described in Section 3.2.2.4; the soluble aggregates in the serum phase were examined in size-exclusion chromatography as described in Section 3.2.2.5. In order to characterise the soluble aggregates in the serum, soluble aggregate fractions were collected between 16 to 17 min, using FRC-10A fraction collector (Shimadzu, USA MFG Inc.). Collected fractions were evaporated to dryness using a GeneVac concentrator. Samples were then analysed by DLS for particle size and \( \zeta \)-potential measurement and by SDS-PAGE as described above.
5.3 Results

5.3.1 Particle size

The casein micelle size in non-sonicated milks increased with increasing pH over the range pH 6.7 – 8.0 (Figure 5.1). This is expected as the negative charge on the proteins increases with pH, resulting in increased electrostatic repulsion between the casein units and swelling of the micelle as indicated previously in Chapter 4. Similar increases were observed in pH-adjusted ultrasonicated milks at 1600 kHz, suggesting that this frequency of ultrasound did not disrupt the casein micelles. In contrast, ultrasonication at 20 kHz or 400 kHz reduced the particle size as pH of the milk was raised. Ultrasonication at 20 kHz caused significant reduction (p < 0.05) of particle size at all pH values and ultrasonication at 400 kHz caused significant reduction (p < 0.05) of particle size at pH 7.5 and 8.0. These results show that the disruptive effects of ultrasonication on casein micelle were increased as the frequency of ultrasonication was reduced and as the pH of the milk was increased (Figure 5.1).
5.3.2 ζ-potential

As shown in Figure 5.2, the ζ-potential of the particles in milk at pH 6.7 was -13.7 ± 0.6 mV. The ζ-potential at natural pH (6.7) is in the range (-8 mV to -25 mV) observed previously by others (Banon & Hardy, 1992; Ekstrand & Larssonraznikiewicz, 1984; Payens, 1966; Wade, Beattie, Rowlands, & Augustin, 1996). The ζ-potential was more negative with increasing pH as expected. There were
no significant changes (p > 0.05) in the measured ζ-potential after the application of ultrasound, irrespective of the frequency of the ultrasound (Figure 5.2). This shows that the particles in both non-sonicated and ultrasonicated milks had similar surface charge.

Figure 5.2 ζ-potential of milks; non-sonicated (●), ultrasonicated at 20kHz (□), 400kHz (▲), or 1600kHz (◇) as a function of pH. Error bars represent standard deviation of triplicates.
5.3.3 Partitioning between the micellar and serum phases

5.3.3.1 Proteins in the serum phase

In milk (10 % TS, pH 6.7), the protein in the serum phase was 22.6 % of the total protein and comprised primarily whey proteins. Increasing pH increased the total protein in the serum of both non-sonicated and ultrasonicated milks (Figure 5.3).

![Figure 5.3](image)

Figure 5.3 Protein content of supernatant obtained from non-sonicated milks (●) and milks ultrasonicated at 20kHz (□), 400kHz (▲), or 1600kHz (◇) at various pH. Error bars represent standard deviation of duplicates.
In particular, increasing pH without the application of ultrasound caused the total protein in the serum of non-sonicated milks to increase from 22.6 % to 31.8 % over the range pH 6.7 to pH 8.0 (Figures 5.3). Ultrasound treatment of milks at pH 6.7 at all frequencies did not significantly alter (p > 0.05) the serum protein concentration (Figure 5.3). However, increasing the pH of milk beyond pH 6.7 prior to sonication increased the protein in the serum of ultrasonicated milks compared to that in non-sonicated milks. The ultrasound-induced increase in the serum protein at higher pH was dependent on the frequency applied, with increased protein in the serum as the frequency was decreased. The highest degree of increase of protein in the serum (51.0 %) was observed in milks at pH 8.0 after ultrasonication of milk at 20 kHz. This was significantly higher than the 44.6 % and 42.1 % observed for milk ultrasonicated at 400 kHz and 1600 kHz, respectively, and 31.8 % for non-sonicated milk (p < 0.05) (Figure 5.3).
Figure 5.4 SDS-PAGE gels (reducing) of non-sonicated milk (pH 6.7) and supernatant from non-sonicated milks (Lanes 1,5,9,13) and milks ultrasonicated at 20 kHz (2,6,10,14), 400 kHz (3,7,11,15) or 1600 kHz (4,8,12,16) at various pH.

Figure 5.4 shows that the increase of protein content in the serum of ultrasonicated milk was due to the release of caseins from the micelles. In all cases, the increase in protein in the serum was due to an increase in concentration of α-, β- and κ-caseins, with little change to α-Lac and β-Lg contents in the serum (Figure 5.4). The absence of low molecular weight proteinaceous material in SDS-PAGE analysis suggests that ultrasound did not result in scission of covalent bonds in the peptide chain. The levels of α-, β- and κ-caseins in serum, expressed as a percentage of each of the individual caseins in non-sonicated milks at each corresponding pH, is shown in Figure 5.5. Individual caseins tended to dissociate in the order of κ- > β- > α-casein, in both non-sonicated and all ultrasonicated milks.
Figure 5.5 Casein in the serum obtained from non-sonicated and ultrasonicated milk. \(\alpha\)-casein (●), \(\beta\)-casein (□) and \(\kappa\)-casein (▲). Error bars represent standard deviation of duplicates.
5.3.3.2 $^{31}$P NMR

$^{31}$P NMR spectra of milk serum from non-sonicated or ultrasonicated milks at pH 8.0 were obtained (Figure 5.6 a). The assignments of the peaks in spectra of the milk serum were based on previous NMR studies of milk (Belton, Lyster, & Richards, 1985). The major peak at 3.25 ppm and the series of minor peaks at 4.1 – 5.2 ppm correspond to Pi and to phosphoseryl (SerP) residues respectively (Ishii, Hiramatsu, Ohba, & Tsutsumi, 2001). The integrated peak areas for Pi and SerP are shown (Figures 5.6 b & c). The SerP peak was significantly increased (p < 0.05) after ultrasonication, especially at low frequency (20 kHz) which suggests an increased release of caseins into the serum (Figure 5.6 b). This is consistent with the information obtained from SDS-PAGE. In contrast, there was no significant change (p > 0.05) in Pi in the serum (Figure 5.6 c), showing that ultrasound did not cause changes to the binding of Pi to the caseins. This suggests that the altered partitioning of caseins into the serum does not involve the dissociation of micellar calcium phosphate.
a

b

Peak area of SerP

Treatments

- non-sonicated
- 20kHz
- 400kHz
- 1600kHz
Figure 5.6 $^{31}$P NMR Analysis (a) Spectra of supernatant obtained from non-sonicated or ultrasonicated milk (pH 8.0), (b) Peak area of SerP and (c) Peak area of Pi. Error bars represent standard deviation of duplicates.
5.3.4 Characteristics of soluble aggregates in serum

To characterise the nature of the soluble aggregates in the serum, the soluble aggregates were fractionated by SEC. The average particle size and the $\zeta$-potential of each fraction were determined, and the protein composition of each fraction was examined using SDS-PAGE.

5.3.4.1 SEC profiles

The SEC profiles obtained from sera of non-sonicated and ultrasonicated milks are shown in Figure 5.7.
Figure 5.7 SE-HPLC profiles of supernatant obtained from non-sonicated (——) milk or milk ultrasonicated at 20kHz (----), 400kHz (-----), or 1600kHz (-------) at various pH. Peak (1) excluded volume, (2) soluble aggregates, (3) whey protein and monomeric casein, (4 - 5) orotic acid and other small solutes.
The peak assignments were based on those reported in previous work (Donato & Dalgleish, 2006; Guyomarc'h, Law, & Dalgleish, 2003; Williams, D'Ath, & Zisu, 2008) and were as follows: Peak 1 (excluded volume of the column), Peak 2 (soluble aggregates), Peak 3 (whey proteins and monomeric caseins) and Peaks 4 & 5 (orotic acid and other small solutes). In non-sonicated milks, the soluble aggregate peak area (Peak 2) increased slightly with increasing pH. Effect of ultrasonication of milk at pH 6.7 or 7.1 was negligible on the SEC profiles. However at higher milk pH (7.5 or 8.0), ultrasonication caused an increase in the soluble aggregate peak area, with the largest increase observed in milk at pH 8.0 after ultrasonication at 20 kHz (Figure 5.7).

5.3.4.2 Particle size, ζ-potential and protein components of soluble aggregates

The average particle size of the soluble aggregates (fraction collected at 16-17 min of Peak 2, Figure 5.7) was 43 nm. There is no information regarding particle size of ultrasound-induced soluble aggregates in milk in the literature.

The ζ-potential of the ultrasound-induced soluble aggregates of non-sonicated and ultrasonicated milks ranged from ~ -13.5 mV (at pH 6.7, non-ultrasonication) to -20.3 mV (at pH 8.0, 20 kHz ultrasonication) (Figure 5.8). As with the ζ-potential of particles in original milk, there was the expected increasing negative charge with increasing pH. The ζ-potential of the soluble aggregates was not significantly different
(p > 0.05) compared to that of the casein micelle in the original milk (Figure 5.8), suggesting that the surface charge of the soluble aggregates formed from the dissociated caseins was similar to that of the casein micelles in the original milk.

Figure 5.8 ζ-potential of non-sonicated milks (●) and soluble aggregates isolated from milks ultrasonicated at 20kHz (□), 400kHz (▲), or 1600kHz (◇) as a function of pH. Error bars represent standard deviation of triplicates.

The SDS-PAGE of the soluble aggregates isolated from non-sonicated and ultrasonicated milk is shown (Figure 5.9). The intensity of the casein bands increased with increasing pH in both non-sonicated and ultrasonicated milks. At a given pH, the
casein band intensities were greatest for the soluble aggregate peak obtained from the serum of milk ultrasonicated 20 kHz, confirming that treatment of milk at 20 kHz increased the formation of soluble aggregates to a greater extent than treatment at 400 kHz or 1600 kHz. The data also showed that only 20 kHz-ultrasound treatment of milk at pH 8.0 resulted in the incorporation of whey proteins into the soluble aggregates (Figure 5.9). The presence of whey proteins in the soluble aggregate fraction is presumably due to the inclusion of denatured whey proteins.

Figure 5.9 SDS-PAGE gels of non-sonicated milk (pH 6.7) and soluble aggregate fraction from non-sonicated milks (Lanes 1,5,9,13) and milks sonicated at 20 kHz (2,6,10,14), 400 kHz (3,7,11,15) or 1600 kHz (4,8,12,16) at various pH.
However, from the SEC data and SDS-PAGE analysis, it was not possible to determine whether the whey proteins in the soluble aggregate fraction were attached to caseins or formed soluble aggregates having the same hydrodynamic radii as the casein aggregates.

### 5.4 Discussion

The particle size and $\zeta$-potential measurements (Figures 5.1 & 5.2) combined with the analysis of proteins in the serum phase (Figures 5.3 – 5.9) provided confirmatory evidence that ultrasound disrupted the casein micelles in milk and that the dissociated caseins re-assembled into smaller particles with similar surface charge. The application of ultrasound (20, 400 and 1600 kHz) at a fixed power (286 kJ/kg) to milk (pH 6.7 – 8.0) did not cause scission of covalent bonds. These observations are in line with other studies which showed that there was no change in primary amine content accompanying the sono-disruption (35 or 135 kHz at 4.1 W per 100 mL for 6 hr, corresponding to 885.6 kJ/kg) of re-assembled casein particles in casein dispersions (Madadlou et al., 2009 b). Changes in primary amine content are only expected if there is hydrolysis of the protein molecule. Neither did ultrasound cause denaturation of whey proteins except in milk at pH 8.0 ultrasonicated at 20 kHz, as shown by the presence of whey proteins in soluble aggregates isolated from this milk (Figure 5.9). This is despite the fact that conditions were chosen to try and avoid heat-induced whey protein denaturation by maintaining the bulk temperature of samples during
ultrasonication below 30 °C. It is possible that the denaturation was due to localised heating caused by the implosion of cavitation bubbles during lower frequency (20 kHz) US processing (Kentish & Ashokkumar, 2011). As the high pH (pH 8.0) favours the denaturation of whey proteins (Law & Leaver, 2000), these two factors may have led to whey protein denaturation.

5.4.1 Effect of pH

When the pH of milk increased from 6.7 to 8.0, the size and the negative charge of the casein particles increased (Figure 5.1). In addition, an increase in the protein content in the serum phase (Figure 5.3) and the release of caseins into the serum (Figures 5.4 & 5.5) were observed with the pH increase of milk. These results are similar to that observed by other workers (Ahmad et al., 2009; Anema & Klostermeyer, 1996, 1997 a, 1997 b; Chandrapala et al., 2011; Liu & Guo, 2008). The alkaline-pH-induced dissociation of caseins from the micelles is believed to be driven by the increased negative charge on the individual caseins which causes swelling of the micelle and alterations to the protein interactions (Ahmad et al., 2009).

Ultrasound application to milk at different pH altered the properties of the milk, with greater effects at higher pH when the same frequency was used. The larger effects of ultrasound at higher pH may be linked to a looser micellar structure as pH is increased, which facilitates transfer of caseins into the serum phase. This result is consistent with
these of others who found that increasing pH increased the disruptive effects of ultrasound on re-assembled casein particles in reconstituted casein powder dispersions (Madadlou et al., 2009 b). An analogous effect, the increased transfer of caseins into the serum phase with increasing pH, has been observed in milk subjected to the stress of elevated temperature (Anema & Klostermeyer, 1996, 1997 b; J. Chandrapala et al., 2011).

### 5.4.2 Effect of ultrasound frequency

The effects of ultrasound were found to increase as the frequency of the ultrasound decreased (at a given milk pH). As the specific energy was kept constant across all frequencies applied, it was possible to separate the effects of specific energy and ultrasound frequency. At low frequency (20 kHz) the changes observed on ultrasound application are primarily due to the physical effects of cavitation, which cause localized shear effects and therefore enhance casein micelle dissociation. However, as the frequency is increased from 20 kHz to 400 kHz and 1600 kHz, the cavitation bubbles become smaller. Therefore the physical force resulting from the implosion of cavitation bubbles is decreased (Ashokkumar, Bhaskaracharya, Kentish, Lee, Palmer & Zisu, 2010; Ashokkumar, Sunartio, Kentish, Mawson, Simons, Vilkhu & Versteeg, 2008). As a result, the effects of shear on casein micelle dissociation will be reduced. As there may be localised increases in temperature when cavitation bubbles implode, it is not possible to unequivocally differentiate between localised temperature effects
induced by ultrasound and shear effects of the acoustic waves *per se*, despite ensuring that the bulk temperature was kept to $< 30$ °C. The chemical effects of cavitation were characterised ([Chapter 3, Section 3.5.2.5](#), Figure 3.6). These results showed that the chemical effects generated at 400 kHz are considerably larger than those at 20 kHz and 1600 kHz, which suggests that chemical effects are not the dominating forces contributing to the micelle disruption observed in this study.

Madadlou *et al.* (2009 a) showed that re-assembled casein particles could be disrupted by applying ultrasound at higher specific energy (35 kHz at 2 – 6.6 W per 100 mL for 6 hr, corresponding to specific energies ranging from 432 – 1426 kJ/kg). These authors observed increased disruption of casein particles with increasing cavitation efficiency when the specific energy was increased at a fixed ultrasound frequency (35 kHz) and suggested that shear forces were responsible for these effects. Our results, which show increased disruption of casein micelles in reconstituted milk with decreasing ultrasound frequency (from 1600 to 20 kHz) at the same specific energy (286 kJ/kg), are in contrast with previously reported effects of ultrasound frequency (35 and 135 kHz at a specific energy of 885.6 kJ/kg) on casein dispersions in phosphate buffer (Madadlou, Mousavi, Emam-Djomeh, Ehsani & Sheehan, 2009b). These authors observed greater disruption of re-assembled casein particles in phosphate buffer by application of ultrasound at 135 kHz compared to 35 kHz when ultrasound was applied at the same specific energy. They suggested that higher concentration of free radicals and greater strain rates resulting from imploding
cavitation bubbles generated by higher frequency were responsible for the greater effects at 135 kHz than at 35 kHz (Madadlou, Mousavi, Emam-Djomeh, Ehsani & Sheehan, 2009b). It is possible that the altered responses to frequency between our work and that of Madadlou et al. (2009b) are due to the differences in the size and nature of the casein micelles in reconstituted milk compared to re-assembled particles in dispersions made with casein powder and phosphate buffer. In reconstituted milk made from low heat powder, the casein micelle largely resembles the native casein micelle in raw milk when the powder is rehydrated (Martin et al., 2007). In milk, the casein micelle is a highly hydrated open particle where the casein molecules are held together in part by nano-clusters of colloidal calcium phosphate (Holt & Horne, 1996). In contrast, the casein particles in the dispersions of casein powder in phosphate buffer used in the work of Madadlou et al. (2009b) are devoid of colloidal calcium phosphate and are larger in size (particle diameter ~ 300 nm at pH 6.7). As previously mentioned, casein powders are conventionally made by acidification of milk to pH 4.6, the isoelectric pH of casein (Augustin, Oliver & Heman, 2011). At this pH, colloidal calcium phosphate is not present in the casein particles (Dalgleish & Law, 1989). When casein powders are dispersed in buffers they will assemble into protein particles because of the natural tendency of the individual casein molecules to self-assemble (Horne, 2002). Consequently our experiments on native-like casein micelles that are present in reconstituted milk described are quite different from the experiments reported by Madadlou et al. (2009 a & b). These differences may have altered the interaction of the particles with the acoustic wave and therefore led to the altered
response of the particles to different frequencies.

5.4.3 Soluble aggregates formed in ultrasonicated milk

This is the first study that reports the composition and characteristics of soluble aggregates formed by processing of milk with ultrasound. The soluble aggregates formed in the serum of milk ultrasonicated at 20 kHz, were comprised primarily of caseins (Figure 5.9), although small amounts of whey proteins were present in the aggregates formed when milk was ultrasonicated at pH 8.0. This contrasts with the soluble aggregates formed as a result of heat processing which are comprised mainly of β-Lg, α-Lac and κ-casein linked through thiol-disulfide bonds in the mass ratio of 1-5 whey protein to 1 κ-casein (Donato & Guyomarc'h, 2009).

It appears that the caseins released from the micelles re-associated in the serum phase to form aggregates which were smaller than the original casein micelles, but which have similar surface charge (Figure 5.8), and without affecting the partitioning of Pi between the micellar and serum phase (Figure 5.6). The size of the ultrasound-induced soluble aggregates (43 nm) is within the range found for heat-induced aggregates (40 - 140 nm) obtained in heated milk (Donato & Dalgleish, 2006; Jean, Renan, Famelart, & Guyomarc'h, 2006; Vásbinder, Alting, & De Kruif, 2003; Vásbinder & De Kruif, 2003). The ζ-potentials of soluble aggregates in ultrasonicated milk (-13.5 ± 0.4 to -20.3 ± 1.2 mV over the pH range 6.7 to 8.0) are
not markedly different from those of heat-induced soluble aggregates (-15 to -20 mV at pH 6.7 to pH 7.0) (Donato & Guyomarc'h, 2009; Jean et al., 2006).

The observation that the re-assembled aggregates carry the same charge as the original micelles is not surprising due to the natural self-assembly behaviour of the caseins. It is generally considered that κ-casein largely locates to the surface of the native casein micelle during their natural assembly (Horne, 2002) through driving forces for the self-assembly of caseins, as has been previously discussed (Holt, 1992; Horne, 2006). It is therefore likely that re-formed casein particles will also have a surface predominantly covered by κ-casein and hence have a similar surface charge. The whey proteins present in the soluble aggregate fraction of milk ultrasonicated at 20 kHz at pH 8.0 did not apparently alter the assembly characteristics of the dissociated casein. Taken together these observations suggest that the dissociated proteins re-assemble to form micelles in a similar fashion to that in the original milk. It is expected that, as in a normal casein micelle, the calcium phosphate will act as a nucleus around which caseins are then attached and therefore be integrated into newly formed particles.
5.5 Conclusion

Ultrasound processing of milk disrupts casein micelles causing release of protein from the micellar to the serum phase and the re-assembly of the protein particles in milk. Casein micelles govern many technological functions of milk such as cheese-making, yoghurt-making and milk powder production. Therefore, it may be expected that ultrasound treatment may be used to introduce novel functionality in milk. Further work is required to explore the novel functionality of ultrasonicated milk.
Chapter 6

Ultrasound processing effects on high-heat treated reconstituted skim milk

Summary

Reconstituted skim milks (10 % w/w total solids) were heat treated at 90 °C for 10 min at various pH (6.3, 6.7, 7.1 and 7.5) and subsequently subjected to 20 kHz ultrasound (US) processing. Dynamic light scattering (DLS) measurements showed that the particle size in heated milks decreased after US treatment. There was no change in ζ-potential of heated milks after US treatment. Nitrogen content measurement of the serum of heated milks showed that the total protein content changed, but $^{31}$P NMR analysis showed that the phosphoserine residues (SerP) were not changed significantly in the serum phase of heated milks after US treatment. SDS-PAGE analysis showed that the increase in protein content in serum was due to the increase in $\alpha$-, $\beta$-, $\kappa$-caseins and $\beta$-lactoglobulin. The soluble aggregates in milk serum were examined by size exclusion chromatography (SEC). SEC results showed that the concentration of the soluble aggregates was higher in heated milks after US treatment, with the most marked effects at pH 6.7. In addition, the soluble aggregate fractions were isolated for further analysis by DLS and SDS-PAGE. DLS measurements showed that the size of soluble aggregates in heated milks was reduced.
by US treatment, while there was no marked change in the ζ-potential of the soluble aggregates. SDS-PAGE gels showed that the composition of the soluble aggregates in heated milks was altered by US treatment.

6.1 Introduction

As discussed in Chapter 2, high-heat treatments are commonly used in dairy industry to achieve desired functionality of milk proteins. The high-heat treated milk protein system has profound technological values in the manufacture of dairy products and influences yoghurt quality, cheese yield and the heat stability of milk (Banks, 1990; Kudo, 1980; Lucey, Teo, Munro, & Singh, 1997). Therefore, the interaction between heat and milk proteins is of great interest and has been extensively studied (Guyomarc’h., Law, & Dalgleish, 2003; Jang & Swaisgood, 1990; Oldfield, Singh, Taylor, & Pearce, 1998; Oldfield, Singh, Taylor, & Pearce, 2000; Smits & Van Brouwershaven, 1980). The physicochemical properties of the milk protein system in high-heat treated milk (85-90 °C × 5-15 min) are also well-defined (Donato & Guyomarc’h, 2009).

Ultrasound, as a novel processing technology, has also been shown to modify milk proteins (Chapter 5) and thus has the potential to alter milk functionalities (Chapters 2 & 7). Future applications of ultrasound processing in the dairy industry may involve integration of US into current dairy processing schemes involving high-heat treated milk with a view to improving milk functionalities or even creating new ones. Thus
the interaction between ultrasound processing and the milk protein system in high-heat treated milk should be examined and understood. In this chapter, the effects of 20 kHz ultrasound processing on the physicochemical properties of milk proteins in high-heat treated milk are investigated.

6.2 Experimental methods

Reconstituted skim milks (10 % TS) at pH 6.3, 6.7, 7.1 and 7.5 were prepared as described in Section 3.1.2. Milks were high-heat treated (90 °C/10 min) as described in Section 3.4. High-heat treated milks were subsequently subjected to ultrasonication at 20 kHz. Milk (100 mL) was ultrasonicated for 9 min in the 20 kHz (Hielscher) US processing system to achieve a specific energy input 286 kJ/kg as described in Section 3.5.2. The bulk temperature of the milk during US processing was controlled < 30 °C as shown in Section 3.5.2.4, Figure 3.5.

The control milks without heat or ultrasonication will be named “untreated milks”. The high-heat treated milks will be named “heated milks”. The ultrasonicated high-heat milks will be named “HUS milks”.

The particle size in milks and the ζ-potential of the particles in milks were measured by dynamic light scattering using a Malvern zetasizer as described in Section 3.2.1.1. Milk serum was obtained by centrifugation as described in Section 3.2.2.1. The
following analysis was carried out in milk serum: protein content was measured using a Leco FP200 as described in Section 3.2.2.2; SDS-PAGE under reducing conditions was performed as described in Section 3.2.2.3; the phosphoseryl residues (SerP) and inorganic phosphorus (Pi) in milk serum were examined by $^{31}$P NMR as described in Section 3.2.2.4; SEC was used to examine soluble aggregates in the serum phase as described in Section 3.2.2.5. In order to characterise the soluble aggregates in the serum, soluble aggregate fractions were collected between 12 and 18 min for samples at pH 6.7, between 12 and 19 min for samples at pH 7.1, between 12 and 20 min for samples at pH 7.5, using FRC-10A fraction collector (Shimadzu, USA MFG Inc.). Collected fractions were evaporated to dryness using a GeneVac concentrator. Samples were then analysed by DLS for particle size and $\zeta$-potential measurement and by SDS-PAGE as described above.

6.3 Results

6.3.1 Particle size and $\zeta$-potential of particles in milk

The mean particle size in untreated milk at pH 6.7 was $198 \pm 3.5$ nm (Figure 6.1) which is in agreement with that previously reported in Chapter 5. It is also in line with previous reports in the literature (Anema, 2007; Holt, 1975; Wade, Beattie, Rowlands, & Augustin, 1996).
In the pH range of 6.3 – 7.5, the mean particle size in untreated milks increased with increasing pH. As previously indicated in Chapter 5, this is considered to be due to the swelling of casein particles caused by the increased electrostatic repulsion between caseins at higher pH (Ahmad, Piot, Rousseau, Grongnet, & Gaucheron, 2009; Liu & Guo, 2008). As shown in Figure 6.1, heat treatment of milk at pH 6.7 did not significantly change the particle size (p > 0.05). When the pH prior to heating was increased to 7.1 or 7.5, heat treatment significantly reduced the particle size (p < 0.05).
However, at pH 6.3, heat treatment significantly increased the particle size (p < 0.05). The increase of particle size by heating milk at pH < 6.7 and the decrease of particle size by heating milk at pH > 6.7 have been reported by other authors (Anema & Li, 2003b; Anema, Lowe & Lee, 2004). The change in particle size is correlated to the association of heat-induced whey protein aggregates with the casein micelles (Anema, Lowe & Lee, 2004).

Heated milks were subjected to 20 kHz US processing. The pH of the milk was measured at room temperature before heating and after US processing and was found little change. After US processing, the particle size of milk heated at pH 6.3 was returned to that of untreated milk at pH 6.3. At pH 6.7, ultrasonication significantly decreased the particle size in heated milk. At pH 7.1, ultrasonication of the heated milk induced a further significant decrease of the particle size (p < 0.05). Ultrasonication of heated milk at pH 7.5 only slightly reduced the particle size and the decrease was not significant (p > 0.05). These results suggest that ultrasonication can disrupt the particles in heated milks at pH 6.3, 7.1 and 7.5 thus causing the particle size reduction.

The ζ-potential in untreated milk at pH 6.7 was -12.9 mV (Figure 6.2). The absolute value slightly decreased at the lower pH (6.3) and slightly increased at the higher pHs (7.1 and 7.5). These changes in the magnitude of the ζ-potential indicated that the particles were more likely to aggregate at lower pH than at the higher pHs. This is
consistent with the expected increase in surface charge at higher pH and is in line with the results previously reported in Chapter 5. The measured values are also within the range of -8 to -25 mV reported in other studies (Banon & Hardy, 1992; Ekstrand & Larssonraznikiewicz, 1984; Payens, 1966; Wade et al., 1996). In the entire pH range of 6.3 to 7.5, neither heating nor heating followed by US had any significant (p > 0.05) effect on the ζ-potential of treated milks.

Figure 6.2 ζ-potential of milk; untreated (■), Heated at 90 °C/10 min (●), Heated at 90 °C/10 min and subsequent 20 kHz ultrasonication (▲). Error bars represent standard deviation of duplicates.
6.3.2 Partitioning between the micellar and serum phases

6.3.2.1 Proteins in the serum phase

The serum protein as a percentage of the total protein in each milk, is shown in Figure 6.3. In untreated milk at pH 6.7, the serum protein content was ~ 24% of the total protein, which is consistent with previous reports. As pH was increased from 6.3 to 7.1 to 7.5, the serum protein content increased slightly in untreated milks. When heat treatment was applied to pH-adjusted milks, serum protein content increased when the pH was increased from 6.7 to 7.1 to 7.5, but decreased at pH 6.3. This is in line with the results of other authors (Chandrapala, Augustin, McKinnon, & Udabage, 2010) who also analysed serum protein content in heated milks at different pHs.

In current study, the heated milks were subsequently ultrasonicated. After ultrasonication of the heated milks, the serum protein content increased at all pHs compared to that in heated milks, indicating a US-induced change in protein distribution between micellar and serum phases at all tested pHs.
Analysis of the protein components in the serum phase by SDS-PAGE showed that at pH 6.3, the increase in serum protein content in HUS milk was mainly due to a slight increase in α-, β-, κ-caseins and β-Lg (Figure 6.4, Lanes 2 & 3). At pH 6.7, the serum protein levels of α-, β-, κ-caseins and β-Lg in HUS milk (Figure 6.4, lane 6) were considerably increased compared to those in heated milk (lane 5). At pH 7.1 and 7.5, serum casein content (α-, β-, κ-caseins) in HUS milks (lane 9 & lane 12 respectively) was markedly increased compared to those in heated milks (lane 8 & lane 11).
respectively), although whey proteins did not appear to change. This clearly
demonstrates that heat treatment and subsequent ultrasonication introduced more
proteins into the serum compared to the heat treatment alone. The change in protein
content in the serum of heated milk after ultrasonication was most marked at pH 6.7.

Figure 6.4 SDS-PAGE gels (reducing conditions) of untreated milk (pH 6.7) and sera of
milks: Untreated (Lanes 1, 4, 7, 10); Heated at 90 °C/10 min (Lanes 2, 5, 8, 11); Heated
at 90 °C/10 min and subsequent 20 kHz ultrasonication (3, 6, 9, 12) at various pH.

6.3.2.2 Phosphorus in serum phase

$^{31}$P NMR spectra were obtained to examine the phosphorus in serum phase. A typical
$^{31}$P NMR spectrum of milk serum at pH 6.7 is shown in Figure 6.5 a. The major peak
at chemical shift 2.2 ppm and the series of peaks at 3.8 – 4.5 ppm were assigned to
inorganic phosphate (Pi) and to phosphoseryl residues (SerP) respectively, based on a previous NMR study of milk (Belton, Lyster, & Richards, 1985).

The major peak areas for Pi and SerP in sera of different milks were determined and are shown in Figures 6.5 b & c. With the increase in pH, the SerP in serum increased in untreated milks. Heat treatment did not have significant change (p > 0.05) on the SerP in serum in milks at any pH. Subsequent ultrasonication of heated milks did not significantly change the SerP content at any pH. Pi content in the serum was also not altered significantly (p > 0.05) by any treatment (Figure 6.5 c). The lack of change in Pi suggests that the calcium phosphate equilibrium in heated milks was not disturbed by ultrasonication.
Figure 6.5 $^{31}$P NMR Analysis (a) Spectra of serum obtained from untreated milk (pH 6.7), (b) Peak area of SerP and (c) Peak area of Pi obtained from sera of milks; Untreated (●), Heated at 90 °C/10min (■), Heated at 90 °C/10min and subsequent 20 kHz ultrasonication (▲). Error bars represent standard deviation of duplicates.

6.3.3 Heat-induced soluble aggregates in milks

6.3.3.1 Size exclusion chromatography

SEC profiles of sera of milks were obtained and are shown in Figure 6.6. As previously discussed in Chapter 5, the peak assignments based on previous studies (Donato & Dalgleish, 2006; Guyomarc'h et al., 2003; Williams et al., 2008) were: peak 1 (excluded volume of the column), peak 2 (soluble aggregates), peak 3 (whey
proteins) and peaks 4 & 5 (orotic acid and other small solutes). In the sera of heated milks (dash dot line) at all pHs, peak 3 essentially disappeared as a result of whey protein denaturation by heat (Williams et al., 2008). After heat treatment at pH 6.7, 7.1 and 7.5, the denatured whey proteins formed aggregates which significantly increased the area of peak 2 (Donato & Dalgleish, 2006). The area increased as the pH increased from 6.7 to 7.1 to 7.5, which indicates that there were more heat-induced soluble aggregates in the serum phase at higher pH. Moreover, peak 2 shifted towards a later elution time with the increase in pH from 6.7 to 7.1 to 7.5. This suggests the heat-induced soluble aggregates formed at higher pH have a smaller size. Similar pH effects on the peak area and the elution time of soluble aggregates were also observed by Donato and Dalgleish (2006). At pH 6.3, heat treatment did not change the soluble aggregate peak (peak 2) which is also in line with the observations of Donato and Dalgleish (2006). Vásbinder and De Kruijf (2003) have suggested that at low pH (pH 6.3) most of the aggregates are bound to the micellar surface and do not appear in the serum phase.
Figure 6.6 Size-exclusion chromatography profiles of sera obtained from milks; Untreated (---), Heated at 90 °C/10 min (-----), Heated at 90 °C/10 min and subsequent 20 kHz ultrasonication (-----) at various pH. Peak (1) excluded volume, (2) soluble aggregates, (3) monomeric whey proteins, (4 - 5) orotic acid and other small solutes.

The effects of HUS (ultrasonication after heating) on the serum SEC profile were also pH dependent. At pH 6.7, 7.1 and 7.5, ultrasonication of heated milks increased the area of peak 2 (dash line). The largest increase (~ 50 %) in peak 2 was at pH 6.7.
These results suggest that ultrasonication of milk heated at these pH values caused the release of heat-induced soluble aggregates into the serum phase of the heat-treated milk. In addition, peak 2 in sera of HUS milks (pH 6.7, 7.1 and 7.5) slightly shifted towards a later elution time. This shift indicates that ultrasonication reduced the size of the heat-induced soluble aggregates in the heated milks.

6.3.3.2 Characterisation of soluble aggregates

In order to study and compare the physicochemical properties of the soluble aggregates, the soluble aggregate fraction (peak 2) in the sera of heated milks and HUS milks (pH 6.7, 7.1 and 7.5) was separated and collected by SEC. The size, ζ-potential and composition of the soluble aggregates were determined.

Particle size analysis (Figure 6.7 a) showed that the average diameter of soluble aggregates in sera of heated milks at pH 6.7, 7.1 and 7.5 was ~ 83, 62 and 45 nm respectively. These sizes are within the range (~ 40 – 140 nm) observed by other authors for heated milk (Donato & Dalgleish, 2006; Jean, Renan, Famelart, & Guyomarc'h, 2006; VASbinder, Alting, & De Kruif, 2003; VASbinder & De Kruif, 2003).
Figure 6.7 Particle size and ζ-potential of soluble aggregates. (a) particle size of soluble aggregates in sera of milks at various pH, (b) ζ-potential of soluble aggregates in sera of milks at various pH; Heated at 90 °C/10 min ( ), Heated at 90 °C/10 min and subsequent 20 kHz ultrasonication ( ). Error bars represent standard deviation of duplicates.

In addition, the decrease in size with the increase in pH from 6.7 to 7.1 to 7.5 was in agreement with previous studies (Donato & Dalgleish, 2006; Renan et al., 2006) which reported that the size of heat-induced soluble aggregates in the serum phase had an inverse relationship with the milk pH (6.3 to 7.3) prior to heating.

When heating was followed by ultrasonication, the size of heat-induced soluble
aggregates in the sera of HUS milks decreased significantly \((p < 0.05)\) when the pH was 6.7 and pH 7.1. At pH 7.5, the heat-induced soluble aggregate size was also reduced by ultrasonication, although the magnitude was much lower compared to those at pH 6.7 and 7.1 (Figure 6.7 a). This is consistent with the shifts in elution time for Peak 2 noted earlier. There was no significant change \((p > 0.05)\) in \(\zeta\)-potential of the heat-induced soluble aggregates after ultrasonication at any pH (Figure 6.7 b), indicating that the stability of the smaller particles produced after ultrasonication was similar to those produced by heat treatment alone.

The composition of the soluble aggregates is shown in Figure 6.8. The intensities of the major protein bands of the soluble aggregates were integrated and are presented in Table 6.1. In heated milk at pH 6.7, the soluble aggregates in the serum phase comprised mainly \(\kappa\)-caseins, \(\beta\)-Lg, \(\alpha\)-Lac, BSA and a small amount of high molecular weight serum proteins as shown in Figure 6.8 (lane 1). Subsequent ultrasonication slightly increased the \(\alpha\)-, \(\beta\)-, \(\kappa\)-casein and \(\beta\)-Lg in the soluble aggregates (Figure 6.8 lane 2 & Table 6.1). At pH 7.1, the \(\alpha\)-, \(\beta\)-, and \(\kappa\)-casein content in the soluble aggregates in HUS milk increased significantly \((p < 0.05)\) (Figure 6.8 lane 4 & Table 6.1), compared to those in heated milk (Figure 6.8 lane 3 & Table 6.1). Small increases in \(\beta\)-Lg were also observed pH 7.1. At pH 7.5, the \(\alpha\)- and \(\beta\)-casein in the aggregates in HUS milk were significantly increased \((p < 0.05)\) compared to those in heated milk (Table 6.1) whereas the content of \(\kappa\)-caseins and whey proteins did not change significantly \((p > 0.05)\). These results show that ultrasonication can modify the
composition (mainly increases in α-, β-casein) as well as the size of soluble aggregates in heated milks.

Figure 6.8 SDS-PAGE gel of untreated milk (pH 6.7) and soluble aggregates obtained from sera of treated milks: Heated at 90 °C/10 min (Lanes 1, 3, 5); Heated at 90 °C/10 min and subsequent 20 kHz ultrasonication (2, 4, 6) at various pH.
Table 6.1 Intensity (arbitrary unit) of protein bands in soluble aggregates of milks at various pH. (Obtained from the integration of band intensity in Figure 6.8).

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH 6.7 Heated</th>
<th>pH 6.7 HUS</th>
<th>pH 7.1 Heated</th>
<th>pH 7.1 HUS</th>
<th>pH 7.5 Heated</th>
<th>pH 7.5 HUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-casein</td>
<td>0</td>
<td>2.53±0.74</td>
<td>4.53±0.18</td>
<td>17.82±6.18</td>
<td>28.60±6.13</td>
<td>42.57±2.18</td>
</tr>
<tr>
<td>β-casein</td>
<td>0</td>
<td>6.99±0.84</td>
<td>12.15±0.48</td>
<td>19.35±2.41</td>
<td>24.96±1.84</td>
<td>30.81±2.03</td>
</tr>
<tr>
<td>κ-casein</td>
<td>47.93±2.38</td>
<td>49.16±2.04</td>
<td>49.00±0.04</td>
<td>62.97±1.23</td>
<td>62.18±6.46</td>
<td>62.56±4.43</td>
</tr>
<tr>
<td>β-Lg</td>
<td>85.51±3.73</td>
<td>87.76±0.45</td>
<td>95.89±2.29</td>
<td>113.93±11.98</td>
<td>90.14±4.84</td>
<td>92.41±15.35</td>
</tr>
<tr>
<td>α-Lac</td>
<td>34.00±1.29</td>
<td>35.75±1.12</td>
<td>39.50±2.45</td>
<td>36.02±1.11</td>
<td>36.80±1.78</td>
<td>35.56±1.90</td>
</tr>
</tbody>
</table>
6.4 Discussion

6.4.1 Ultrasound interferes with the partitioning of aggregates between micellar and serum phases in heat-treated milk

The distribution of heat-induced whey protein/κ-casein aggregates between the micellar and serum phases in heat-treated milk is highly dependent on the pH of the milk prior to heat treatment. In the pH range of 5.8 to 8.5, studies from different authors have consistently indicated that more aggregates are formed on the surface of casein micelles after heat treatment at lower pH, while more aggregates are formed in the serum at higher pH values (Anema, 2007; Anema & Klostermeyer, 1997b; Chandrapala et al., 2010; Corredig & Dalgleish, 1996; Donato & Dalgleish, 2006; Oldfield et al., 2000; Renan et al., 2006; Smits & Van Brouwershaven, 1980; Vasbinder & De Kruif, 2003).

In this study, when ultrasonication was applied to heated milks, the mean particle size in the milk decreased (Figure 6.1). The particle size reduction caused by ultrasonication may be due to the detachment of the whey protein/κ-casein aggregates from the micelles and the dissociation of micellar caseins. This argument is supported by the evidence that ultrasonication increased levels of both whey proteins and caseins in the sera of heated milks, albeit to different extents depending on the milk pH (Figure 6.4). At pH 6.7, ultrasonication introduced more β-Lg and α-Lac into the
serum of heated milk, presumably by detaching the heat-induced aggregates from the casein micelles. The formation of these micellar aggregates in heated milk is due to the denatured whey proteins (mainly β-Lg and α-Lac (Noh, Richardson, & Creamer, 1989; Oldfield et al., 2000)) associating with micellar κ-caseins through hydrophobic and thiol-disulphide exchanges (Jang & Swaisgood, 1990; Jean et al., 2006; Lowe et al., 2004; Oldfield, Singh, & Taylor, 1998; Singh & Creamer, 1991). In Chapter 5 (Section 5.3.2.1, Figure 5.6), it has showed that 20 kHz ultrasonication can cause the dissociation of micellar caseins (α-, β, and κ-caseins) into the serum phase in (unheated) milk at pH 6.7, which is attributed to the strong physical forces generated by ultrasound at low frequency. The sonication-induced dissociation of κ-caseins from casein micelles was also observed by Nguyen and Anema (2010). Hence, the detachment of the whey protein/κ-casein aggregates from the micelle surface may be due to the dissociation of κ-caseins that have been associated with denatured whey proteins.

At pH 7.1 and 7.5, although subsequent ultrasonication still slightly increased the κ-casein and β-Lg in the sera of heated milks (Figure 6.4, lanes 9 & 12), presumably by detaching the heat-induced whey protein/κ-casein aggregates from the micelle surface, this effect was not as marked as that at pH 6.7 (Figure 6.4, lane 6). This is because at pH 7.1 and 7.5, most of the heat-induced whey protein/κ-casein aggregates were already present in the serum phase (Figure 6.4, lanes 8 & 9) rather than on the micelle surface. On the other hand, at pH 7.1 and 7.5, more α-, β- and κ-caseins were
dissociated to the serum phase by subsequent ultrasonication of heated milks (Figure 6.4, lanes 9 & 12), compared to those at pH 6.7 (lane 6). These caseins may form small micelle-like aggregate particles and become part of the soluble aggregates in the serum, which also explains the lack of change in SerP in the serum of HUS milks, compared to that of heated milks (Figure 6.6 b).

Vasbinder and De Kruif (2003) observed that heat treatment of milk induced about 50 %, 60 % and 80 % of whey aggregates associating with casein micelles at pH 6.7, 6.55 and 6.35 respectively. The results reported in this study (Figures 6.3, 6.4 & 6.6) also suggest that, in heated milk at pH 6.3, most of the whey protein/\kappa-casein aggregates were formed on the micelle surface. As a result, the casein micelle size significantly increased (Figure 6.1). Subsequent ultrasonication of milk heated at pH 6.3 reduced the particle size back to the size before heat treatment was applied. This may be due to that ultrasonication substantially detached the aggregates from the micelle surface back into the serum. However, it should be noted that the level of serum proteins (Figures 6.3 & 6.4) and the soluble aggregate peak area (peak 2) (Figure 6.6) in the serum did not significantly increase in HUS milk at pH 6.3. It is most likely that the detached aggregates formed after HUS at pH 6.3 were not truly soluble and sedimented with the casein micelles on centrifugation.
6.4.2 Modification of the properties of heat-induced protein aggregates by ultrasound

Ashokkumar et al. (2009) have shown that 20 kHz ultrasonication can reduce the size of whey protein aggregates. They produced these aggregates by heating whey protein retentate solutions at 80 °C for 1 min. These authors speculated that cavitation shear forces were responsible for disrupting hydrophobic interactions and/or disulfide bonds between the denatured whey protein molecules. Although our investigation of ultrasound effects on milk proteins was undertaken in heat-treated milk, a medium with much more complex chemical and physical properties than whey protein retentate solution, ultrasonication was also able to reduce the size of the soluble aggregates in the serum phase (Figure 6.7 a). The physical forces generated by ultrasound at low frequency have been shown to be strong enough to break polymer chains (Price & Smith, 1993), degrade dextranes (Portenlänger & Heusinger, 1997), break aluminium oxide particles (Raman & Abbas, 2008) or reduce the size of alumina and silica particles (Lu, Riyanto, & Weavers, 2002). The reduction of the soluble aggregate size reported here may also be due to the strong physical effects of cavitation breaking the bonds in the aggregates. In addition, the extent of particle size reduction is larger at lower pH (Figure 6.7 a), where the initial size of the aggregates was larger. Different studies have shown that heat-induced aggregates in milk serum generally have a spherical shape (Creamer, Berry, & Matheson, 1978; del Angel & Dalgleish, 2006; Jean et al., 2006). The physical forces of ultrasound such as shear forces, microjets and shock waves may be mostly exerted on the surface of the
aggregate particles. Thus larger particles with higher surface areas may be more susceptible to the cavitation physical forces and consequently have a higher size reduction.

6.5 Conclusion

High intensity, low frequency (20 kHz) ultrasound processing can detach the heat-induced whey protein/κ-casein aggregates from the surface of casein micelles into the serum phase in reconstituted skim milk. The mean particle sizes of the casein micelles and heat-induced soluble aggregates in the serum of heated milk are also reduced by ultrasound processing. Moreover, ultrasound processing can introduce micellar α- and β-caseins into the heat-induced soluble aggregate fractions thus modifying their composition. The changes of distribution, size and composition of the heat-induced aggregates may have significant effects on the acid-induced gelation properties and heat stability of milk, which will be explored in Chapter 7.
Chapter 7

Functionality of ultrasonicated milks

Summary

The effects of ultrasound processing on selected functional properties of milk were determined. All milks were adjusted to a constant pH 6.7 prior to all the functionality tests. The examined functional properties were renneting properties, viscosity during concentration, acid-induced gelation properties and heat stability. The effects of ultrasound processing on these properties are summarised below.

- The gelation time, gelation rate, curd firmness, and the connectivity of the gel network were improved significantly in rennet gels made from milk ultrasonicated (20 kHz) at pH 8.0, compared to those made from untreated milk at pH 6.7, from untreated milk with pH raised to 8.0 and readjusted to 6.7 and from milk ultrasonicated (20 kHz) at pH 6.7.

- The viscosities of concentrated milk (up to ~ 55 % total solids) obtained on evaporation of milk ultrasonicated (20 kHz) at pH 8.0 were higher compared to those of concentrated milk made from untreated milk at pH 6.7.

- GDL-induced acid gels made from heated (90 °C/10 min) milks that had been ultrasonicated (20 kHz) at pH 6.3, 6.7 and 7.1, had similar properties (gelation pH, gelation time, and gel firmness) compared to those made from heated
milk at corresponding pHs.

- The heat stability of recombined concentrated milks (% total solids : % fat, 26 : 8) made from heated (90 °C/10 min) milk that had been ultrasonicated (20 kHz) at pH 6.7 was no better than the heat stability of those made from untreated milks at pH 6.7 and heated milks at pH 6.7.

7.1 Introduction

The dairy industry processes large amounts of milk into dairy-based products such as cheese, yoghurt, milk powder or evaporated concentrated milk. Many studies have shown that different factors such as gelation temperature or pH, milk solid concentration, milk protein content and Ca content have effects on rennet-induced milk gel properties (Bansal et al., 2007; Choi, Horne & Lucey, 2007; Lucey, Johnson, & Horne, 2003; Mishra et al., 2005; Van Hooydonk, 1987; Zoon, Vanvliet, & Walstra, 1988a, 1988b). The effects of prior heat treatment on acid-induced milk gels have been extensively studied (Horne, 1999; Lucey, 2002; Lucey & Singh, 2003; Lucey, van Vliet, Grolle, Geurts, & Walstra, 1997). However, the effects of ultrasound processing of milk on the rennet-induced and GDL-induced gelation properties are unknown. In addition, it is not known whether ultrasound processing of milk affects the viscosity of milk during the concentration process in milk powder production or the heat stability of recombined concentrated milk. In Chapters 5 and 6, it has been shown that ultrasound processing (particularly 20 kHz ultrasonication) can modify the physicochemical properties and partitioning of milk proteins. The modified milk
proteins may show novel functional properties. In this chapter, selected milks were examined in different functionality tests.

It should be noted that the changes in physicochemical properties and protein partitioning reported in Chapters 5 and 6, were for milks at the pH occurring at the end of the ultrasound treatment (i.e. pH 6.7, 7.1 7.5, 8.0). The functionality tests required the milks to be returned to natural pH (6.7) before testing and it was not known whether the US-induced changes in particle size and protein partitioning would persist when the US-treated milk was returned to pH 6.7. Therefore, in order to indicate the state of the protein system (particularly casein micelles) after the pH re-adjustment (pH to 6.7) in selected milks, the average size and the size distribution of the protein particles were measured after pH re-adjustment.

7.2 Experimental methods

7.2.1 Milk sample selection

Table 7.1 lists the selected milks and corresponding functionality tests. It is important to note that all the selected milks that were processed at pH values other than pH 6.7, were re-adjusted to natural pH 6.7 with 1 M HCl/NaOH before the functionality tests.
Table 7.1 Selected milk samples for functionality tests

<table>
<thead>
<tr>
<th>Functionality tests</th>
<th>Milk samples selected for examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rennet-induced gelation</td>
<td><strong>Selected from Chapter 5:</strong></td>
</tr>
<tr>
<td></td>
<td>1. Untreated milk* at pH 6.7</td>
</tr>
<tr>
<td></td>
<td>2. 20kHz-ultrasonicated milk § at pH 6.7</td>
</tr>
<tr>
<td></td>
<td>3. Untreated milk at pH 8.0</td>
</tr>
<tr>
<td></td>
<td>4. 20kHz-ultrasonicated milk at pH 8.0</td>
</tr>
<tr>
<td>Viscosity during concentration</td>
<td><strong>Selected from Chapter 5:</strong></td>
</tr>
<tr>
<td></td>
<td>1. Untreated milk at pH 6.7</td>
</tr>
<tr>
<td></td>
<td>2. 20 kHz-ultrasonicated milk at pH 8.0</td>
</tr>
<tr>
<td>GDL-induced gelation</td>
<td><strong>Selected from Chapter 6:</strong></td>
</tr>
<tr>
<td></td>
<td>1. Untreated milk at pH 6.3, 6.7, and 7.1</td>
</tr>
<tr>
<td></td>
<td>2. Heated milk ☐ at pH 6.3, 6.7, and 7.1</td>
</tr>
<tr>
<td></td>
<td>3. HUS milk @ at pH 6.3, 6.7, and 7.1</td>
</tr>
<tr>
<td>Heat stability of recombined concentrated milk</td>
<td><strong>Selected from Chapter 6:</strong></td>
</tr>
<tr>
<td></td>
<td>1. Untreated milk at pH 6.7</td>
</tr>
<tr>
<td></td>
<td>2. Heated milk at pH 6.7</td>
</tr>
<tr>
<td></td>
<td>3. HUS milk at pH 6.7</td>
</tr>
</tbody>
</table>

* Untreated milk: milk that was not exposed to any treatment.
§ 20kHz-ultrasonication milk: milk that was ultrasonicated at 20 kHz.
☒ Heated milk: milk that was high-heat treated (90 °C/10 min).
@ HUS milk: milk that was high-heated treated (90 °C/10 min) and subsequently ultrasonicated at 20 kHz.

All milks were re-adjusted back to pH 6.7 before all the functionality tests.

7.2.2 Rennet-induced gelation

The average particle size and particle size distribution of milks were measured using dynamic light scattering (DLS) and size-exclusion chromatography (SEC) respectively, as described in Section 3.2.1.1 and Section 3.2.1.2. The rheological properties of the rennet-induced gels of the milks including curd firmness, gelation time and maximum curd firming rate were measured using the methods described in Section 3.3.1.1. The microstructure of the set rennet-induced gels was examined by
confocal laser scanning microscopy (CLSM) as described in Section 3.3.1.2.

7.2.3 Viscosity of milk during concentration

The viscosity of milks during concentration was measured as described in Section 3.3.2.

7.2.4 GDL-induced gelation

The particle size distribution of milks was measured using size-exclusion chromatography (SEC) as described in Section 3.2.1.2. The rheological properties of GDL-induced gels of selected milks including gelation pH, gelation time, and curd firmness were measured as described in Section 3.3.3.

7.2.5 Heat stability of recombined concentrated milk

The recombined concentrated milks were prepared from selected milks and the heat stability was measured as described in Section 3.3.4.
7.3 Results

7.3.1 Rennet-induced gelation

7.3.1.1 Particle size by DLS

As shown in Figure 7.1, the mean particle size in untreated milk at pH 6.7 was 205 nm. US treatment of milk at pH 6.7 reduced the particle size to 192 nm which is in line with the particle size reduction described in Chapter 5 (Figure 5.1). When untreated milk (pH 6.7) was adjusted to pH 8.0 and re-adjusted to pH 6.7, the mean particle size was 208 nm, which was similar to that in untreated milk at pH 6.7 (205 nm). However, when 20 kHz ultrasound processing was applied to milk at pH 8.0 followed by pH re-adjustment back to 6.7, the mean particle size (≈ 169 nm) was significantly smaller (p < 0.05) than that in untreated milk at pH 6.7.
Figure 7.1 Mean particle diameter of untreated milks at pH 6.7 and 8.0, and milks 20 kHz-ultrasonicated at pH 6.7 and 8.0. All milks treated at pH 8.0 were re-adjusted to pH 6.7 before the measurements. Error bars represent standard deviation of triplicates.

7.3.1.2 SEC profiles

The SEC profiles of the milks used in the rennet-induced gelation experiments are shown in Figure 7.2. Peaks 1 (particle size > 160 nm), 2 (particle size range 162–80 nm) and 3 (particle size range 80–37 nm) represent large, medium and small micelle particles respectively. Peak 4 represents whey proteins. Peaks 5 and 6 represent small
peptides and small aromatic molecules respectively (Williams et al., 2008). The peaks were integrated and their areas are presented in Table 7.2 as the percentage of the total peak area in each milk.

Figure 7.2 Size-exclusion chromatography profiles of milks; Untreated at pH 6.7 (---), Ultrasonicated at pH 6.7 (-- -), Untreated at pH 8.0 and re-adjusted to 6.7 (— —), Ultrasonicated at pH 8.0 and re-adjusted to 6.7 (— —). Particle size (diameter) of eluted materials (■).

Peak 4, 5 and 6 did not change significantly (p > 0.05) regardless of treatments (Table 7.2). The percentages of micelle particles (peaks 1, 2, & 3, Table 7.2) were similar in untreated milk at pH 6.7 and untreated milk adjusted to pH 8.0 and re-adjusted to pH 6.7. Large and medium size micelle particles dominated the micelle particle population. However, ultrasonication of milk at pH 6.7 reduced the population of
large micelle particles (from 30 to 21 %) and slightly increased the population of medium (31 to 37 %) and small (14 to 17 %) micelle particles. In milk ultrasonicated at pH 8.0 and adjusted to pH 6.7, the percentage of large micelles (peak 1, 16 %) was significantly lower (p < 0.05) and the percentage of small micelles (peak 3, 30 %) was significantly higher (p < 0.05), compared to those in untreated milk at pH 8.0 and adjusted to pH 6.7 (peak 1, 30 %; peak 3, 16 %).

Table 7.2 Peak percentages from Figure 7.2. Each figure is the area of the peak expressed as a percentage of total peak area in the corresponding milk

<table>
<thead>
<tr>
<th>% of Area</th>
<th>pH 6.7</th>
<th>pH 8.0and re-adjusted to 6.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>untreated</td>
<td>ultrasonicated</td>
</tr>
<tr>
<td>1</td>
<td>29.8 ± 0.6</td>
<td>20.8 ± 1.9</td>
</tr>
<tr>
<td>2</td>
<td>31.1 ± 0.3</td>
<td>36.8 ± 0.8</td>
</tr>
<tr>
<td>3</td>
<td>14.4 ± 1.6</td>
<td>16.8 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>12.7 ± 0.8</td>
<td>13.5 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>10.3 ± 0.6</td>
<td>10.7 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>1.7 ± 0.1</td>
<td>1.4 ± 0.7</td>
</tr>
</tbody>
</table>

7.3.1.3 Rennet-induced gels

The gelation time, maximum curd firmness, and curd firming rate of rennet-induced milk gels are given in Table 7.3. The milk ultrasonicated at pH 8.0 and re-adjusted to pH 6.7 had the shortest gelation time (28 min), while the untreated milk at pH 6.7 had the longest gelation time (~ 40 min). The maximum curd firmness of milk ultrasonicated at pH 8.0 and re-adjusted to pH 6.7 was 47.10 Pa, which was approximately 7 fold higher than that of gel formed from untreated milk at pH 6.7.
The curd firming rate of the milk ultrasonicated at pH 8.0 and re-adjusted back to pH 6.7 was \( \sim 0.79 \) Pa/min, which was also about 7 fold higher than that of untreated milk at pH 6.7 (0.11 Pa/min). The gelation time, maximum curd firmness and curd firming rate of the gels made from milk ultrasonicated at pH 8.0 and re-adjusted back to pH 6.7 were also significantly superior (\( p < 0.05 \)) to those made from milk ultrasonicated at pH 6.7 and those made from untreated milk adjusted to pH 8.0 and re-adjusted to pH 6.7.

**Table 7.3** Key rennet-induced gelation properties in milks subjected to different treatments

<table>
<thead>
<tr>
<th></th>
<th>pH 6.7</th>
<th>pH 8.0 and re-adjusted to 6.7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Ultrasoundated</td>
</tr>
<tr>
<td>Gelation time (min)</td>
<td>40.00 ± 0.71</td>
<td>35.25 ± 0.35</td>
</tr>
<tr>
<td>Max.curd firmness (Pa)</td>
<td>6.60 ± 0.26</td>
<td>13.90 ± 0.57</td>
</tr>
<tr>
<td>Curd firming rate (Pa/min)</td>
<td>0.11 ± 0.01</td>
<td>0.23 ± 0.01</td>
</tr>
</tbody>
</table>

The curves of the storage modulus (\( G' \)) against renneting time are presented in Figure 7.3 to provide a clear illustration of the differences between this rennet-induced gelation property in milk ultrasonicated at pH 8.0 and re-adjusted to 6.7 and in the other 3 milks examined.

These results demonstrate that ultrasound processing of milk at pH 8.0 (followed by re-adjustment of pH to 6.7) markedly improved the rennet-induced gelation properties of milk.
7.3.1.4 Microstructure of the rennet-induced gels by CLSM

The microstructures of the rennet-induced gels are shown in Figure 7.4. There were much larger pores and much less interconnectivity in the gels of untreated milk at pH 6.7 (Figure 7.4 a) compared to those of the other three milks. The gel matrix of milk ultrasonicated at pH 8.0 and re-adjusted to pH 6.7 (Figure 7.4 d) had the densest structure, as indicated by greater connectivity between the strands and clusters in the network. The pore sizes and interconnectivity of the gels from ultrasonicated milk at
pH 6.7 (Figure 7.4 b) and untreated milk at adjusted to pH 8.0 and re-adjusted to 6.7 (Figure 7.4 c) were similar and in between those gels made from the other two milks (Figures 7.4 a & d).

Figure 7.4 Microstructure of rennet-induced gels of milks: (a) Untreated at pH 6.7; (b) Ultrasonicated at pH 6.7; (c) Untreated at pH 8.0 and re-adjusted to 6.7; (d) Ultrasonicated at pH 8.0 and re-adjusted to 6.7.
7.3.2 Viscosity of milk as a function of concentration

The curves of viscosity as a function of concentration of milks are presented in Figure 7.5. The data was fitted using SigmaPlot (Version 10.0). At low concentration (< ~ 40 % TS), there was little difference between the viscosity of untreated milk at pH 6.7 (■) and that of ultrasonicated milk treated at pH 8.0 and re-adjusted to 6.7 (●). However, at concentrations above 40 % TS, the viscosities of milks treated with US at 8.0 and re-adjusted to 6.7 were higher than those of untreated milks at pH 6.7.

Figure 7.5 Viscosity (at 50 s⁻¹ shear rate) of milks during concentration process; Untreated milk at pH 6.7 (■), milk ultrasonicated at pH 8.0 and re-adjusted to 6.7 (●).
7.3.3 GDL-induced gelation

7.3.3.1 SEC profiles of high-heat treated milks used in GDL-induced gelation

The SEC profiles of untreated and treated milks are shown in Figure 7.6. Peak assignments are as described in Section 7.3.1.2. In heated milks at all pHs, the whey proteins were denatured, as indicated by the disappearance of peak 4. High-heat treatment of milk at pH 6.3 with subsequent pH re-adjustment to 6.7 increased the large micelle population (peak 1). A slightly smaller increase in the large micelle population was also observed in milk heated at pH 6.7. Meanwhile, the medium (peak 2) and small (peak 3) micelle populations also increased in milk heated at pH 6.7. High-heat treatment of milk at pH 7.1 with subsequent pH adjustment to 6.7 slightly decreased the large micelle (peak 1) population and markedly increased the medium and small micelle populations.

Ultrasonication of heated milk at pH 6.3 followed by pH adjustment to 6.7 reduced the large micelle (peak 1) population and increased the medium and small micelle populations. Ultrasonication of heated milk at pH 6.7 also reduced the large micelle population and slightly increased the small micelle population. However, when ultrasonication was applied to milk heated at pH 7.1, the micelle population remained essentially unchanged.
Figure 7.6 Size-exclusion chromatography profiles of untreated milks (---), heated milk (---), and HUS milk (milk that was heated followed by subsequent ultrasonication) (—) at pH (a) 6.3, (b) 6.7, and (c) 7.1. All milks that were not at pH 6.7 were re-adjusted back to pH 6.7 before measurements.

7.3.3.2 GDL-induced acid gels

The curves showing changes in storage modulus (G’) and sample pH with time during gelation are presented in Figure 7.7. The value of tan δ as a function of time is also presented in Figure 7.8. In addition, the gelation time, gelation pH, maximum G’, and tan δ maximum of the milks are summarised in Table 7.3. The pH of the milks with added GDL (1.3 %) dropped to ~ 4.6 after 180 min incubation time at 43 °C (Figure
7.7). The differences in the gelation process between untreated (square symbols) and heated or HUS milks (circle or triangle symbols) are clearly illustrated in Figures 7.7 and 7.8.

Figure 7.7 pH ( ) of milk during GDL-induced gelation. Storage modulus (G') during GDL-induced gelation: Untreated at pH 6.3 and re-adjusted to 6.7 ( ); Heat treated at pH 6.3 and re-adjusted to 6.7 ( ); Heat treated at pH 6.3 followed by US processing and re-adjusted to 6.7 ( ); Untreated at pH 6.7 ( ); Heat treated at pH 6.7 ( ); Heat treated at pH 6.7 followed by US processing ( ); Untreated at pH 7.1 and re-adjusted to 6.7 ( ); Heat treated at pH 7.1 and re-adjusted to 6.7 ( ); Heat treated at pH 7.1 followed by US processing and re-adjusted to 6.7 ( ).
In untreated milks at all pHs, the gelation times (~ 81 – 103 min) were considerably longer, the gelation pH (~ 4.82 – 4.90) were lower, and the max. $G'$ (~ 6.27 – 20.70 Pa) were much lower, compared to the gelation properties of heated or HUS milks. After the start of gel formation in heated and HUS milks, the tan $\delta$ decreased initially but
then increased to a maximum value. High tan δ indicates the increase of susceptibility
of bonds in the gel network to be broken, which can promote the rearrangements of
the gel forming particles (Vanvliet, Vandijk, Zoon, & Walstra, 1991). But this
maximum in tan δ did not occur in untreated milks. This phenomenon was also
observed in previous study (Lucey et al., 1998). However, between the heated milks
and the HUS milks, there were essentially no differences in gelation time, gelation pH,
maximum G’ and maximum in tan δ (Table 7.4). These results indicate that ultrasound
processing did not cause significant changes in GDL-induced gelation properties of
heated milks, despite producing a difference in the particle size of proteins.
Table 7.4 GDL-induced gelation properties of milks subjected to different treatments.

<table>
<thead>
<tr>
<th></th>
<th>pH 6.3 re-adjusted to 6.7</th>
<th>pH 6.7</th>
<th>pH 7.1 re-adjusted to 6.7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Heated</td>
<td>HUS</td>
</tr>
<tr>
<td>Gelation time (min)</td>
<td>96.00 ± 5.66</td>
<td>40.50 ± 6.36</td>
<td>40.00 ± 1.41</td>
</tr>
<tr>
<td>Gelation pH</td>
<td>4.82 ± 0.02</td>
<td>5.03 ± 0.06</td>
<td>4.99 ± 0.05</td>
</tr>
<tr>
<td>Storage modulus, G' (Pa)</td>
<td>7.47 ± 1.60</td>
<td>161.73 ± 9.12</td>
<td>145.31 ± 5.50</td>
</tr>
<tr>
<td>Maximum in tan δ</td>
<td>-</td>
<td>0.47 ± 0.01</td>
<td>0.51 ± 0.01</td>
</tr>
</tbody>
</table>

Gelation time is defined as the point when gels had a $G' \geq 1$ Pa.

G' value is at 180 min for gels formed at 43 °C.

Maximum in tan δ was defined as the point after gelation when the value of tan δ increased to a maximum.

Gelation pH is the pH value of the sample at the gelation time.
7.3.4 Heat stability of recombined concentrated milk

The pH of the recombined concentrated milks was adjusted to values between ~ 6.2 and 6.8. Commercial high-heat stable milk was used to prepare recombined concentrated milk in the heat stability test as a positive control of the sterilization method. As shown in Table 7.5, the viscosities of the commercial high-heat stable milks at pH 6.27 to 6.45 were ~ 24 to 27 mPa.s. But when the pHs of the samples were outside this range, the samples became very viscous and completely gelled after the sterilization heat treatment. The recombined concentrated milks made from untreated, heated and HUS milks, however, gelled after the sterilization heat treatment within the whole tested pH range.

Table 7.5 Viscosity of different recombined concentrated milks at various pH.

<table>
<thead>
<tr>
<th>Commercial high heat stable milk</th>
<th>Untreated milk at pH 6.7</th>
<th>Heated milk at pH 6.7</th>
<th>HUS milk at pH 6.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH Visc</td>
<td>pH Visc</td>
<td>pH Visc</td>
<td>pH Visc</td>
</tr>
<tr>
<td>6.67 Fail*</td>
<td>6.76 Fail</td>
<td>6.78 Fail</td>
<td>6.76 Fail</td>
</tr>
<tr>
<td>6.63 Fail</td>
<td>6.72 Fail</td>
<td>6.72 Fail</td>
<td>6.70 Fail</td>
</tr>
<tr>
<td>6.56 Fail</td>
<td>6.67 Fail</td>
<td>6.66 Fail</td>
<td>6.64 Fail</td>
</tr>
<tr>
<td>6.50 71.9</td>
<td>6.65 Fail</td>
<td>6.61 Fail</td>
<td>6.57 Fail</td>
</tr>
<tr>
<td>6.45 24.62</td>
<td>6.59 Fail</td>
<td>6.55 Fail</td>
<td>6.52 Fail</td>
</tr>
<tr>
<td>6.37 26.85</td>
<td>6.53 Fail</td>
<td>6.48 Fail</td>
<td>6.46 Fail</td>
</tr>
<tr>
<td>6.32 26.04</td>
<td>6.47 Fail</td>
<td>6.44 Fail</td>
<td>6.41 Fail</td>
</tr>
<tr>
<td>6.27 27.45</td>
<td>6.45 Fail</td>
<td>6.38 Fail</td>
<td>6.35 Fail</td>
</tr>
<tr>
<td>6.22 Fail</td>
<td>6.39 Fail</td>
<td>6.34 Fail</td>
<td>6.31 Fail</td>
</tr>
</tbody>
</table>

*Samples that were not pourable were classed as “Fail”.
7.4. Discussion

7.4.1 Effects of US on the renneting properties of milk

There are no reports about the renneting properties of ultrasonicated milk attributed to the ultrasound effects on casein micelles. In this chapter, it has been shown that the rennet-induced gelation properties of milk ultrasonicated at pH 8.0 and re-adjusted to 6.7 were superior to those of the other 3 milks (untreated milk at pH 6.7, milk ultrasonicated at pH 6.7, and untreated milk at pH 8.0 and re-adjusted to 6.7), as indicated by the lower gelation time, higher curd firmness, and curd firming rate (Table 7.3) and a denser microstructure with more interconnectivity (Figure 7.4). This significant improvement of the renneting properties was attributed to the modification of casein micelle particles by US processing.

In this chapter, Figure 7.1 showed that the ultrasound-induced reduction of particle size, observed in ultrasonicated milk at pH 8.0, was maintained after the pH was adjusted back to 6.7 for use in functionality tests. It was found that the particle size of milk ultrasonicated at pH 8.0 and re-adjusted to 6.7 was ~ 35 nm smaller compared to that in untreated milk at pH 8.0 and re-adjusted to 6.7 (Figure 7.1). This echoed the results presented in Chapter 5 where we showed that ultrasonication (20 kHz) of milk at pH 8.0 can disrupt the casein micelles and reform new micelles with smaller particle size (~ 35 nm smaller) (Section 5.3.1, Figure 5.1).
Furthermore, small particles were found to be the dominating population in milk that was ultrasonicated at pH 8.0 and re-adjusted to 6.7 (Figure 7.2 & Table 7.2). These results are consistent with the observation in Chapter 5 that ultrasonication at pH 8.0 created a considerable amount of micelle-like small particles in the serum phase (Section 5.3.3, Figure 5.7). The SEC profiles (Figure 7.2) show that the micelle particle size distribution was dominated by the large micelle particle (peak 1) and medium micelle particle (peak 2) populations in untreated milk at pH 6.7, untreated milk at pH 8.0 that was re-adjusted to 6.7, and milk ultrasonicated at pH 6.7. In contrast, the small micelle (peak 3) population in milk ultrasonicated at pH 8.0 and re-adjusted to 6.7 was ~ 2 fold larger (Table 7.2) than corresponding fractions in the other milks. The particle size in the small-micelle population was calculated to be in the range of ~ 37 – 80 nm (Figure 7.2) which encompasses that of the micelle-like particles (average diameter 43 nm) found in the serum phase after ultrasonication of milk at pH 8.0 (Chapter 5, Section 5.3.3, Figure 5.7). This suggests that the partitioning changes, observed in ultrasonicated milk at pH 8.0, were preserved when the pH was re-adjusted back to 6.7 prior to functionality tests.

Thus it is reasonable to conclude that, when US processing disrupted casein micelles, the released caseins reformed into smaller micelle particles which persisted after pH re-adjustment to 6.7, dominated the particle size population and subsequently resulted in modification of the renneting properties.
As caseins form the matrix of the curd, casein micelle size is of great importance to the gelation characteristics. It has been shown that stronger rennet-induced gels were formed in milk with smaller native casein micelles (Glantz et al., 2010). Walsh et al. (1995) studied the genetic variants of caseins and observed that κ-casein BB milk had smaller micelles compared to AA milk. The rennet coagulation properties such as gelation time, gel strength and firmness were superior in the κ-casein BB milk. The majority of previous studies also indicated that milk with smaller casein micelles showed higher rennet-induced gelation rate, shorter gelation time or higher gel strength (Ford & Grandison, 1986; Niki et al., 1994; Niki, Kohyama, Sano, & Nishinari, 1994). The proportion of κ-caseins in a smaller casein micelle is higher than that in a bigger casein micelle (Ekstrand & Larssonraznikiewicz, 1978). Moreover, samples consisting of smaller micelles have a larger total surface area compared to those consisting of larger micelles. As the κ-caseins are mainly located on the surface of the micelles (Waugh & Talbot, 1971), the larger surface area and higher proportion of κ-caseins in small casein micelles favour the enzymatic activity of rennet on κ-caseins. Therefore the gelation starts earlier with a higher rate, giving more time for the interaction of destabilized casein micelles to form a gel network. In addition, the small size of the micelles may allow a more compact arrangement of the gel-forming particles which can lead to more inter-micelle bonds and consequently formation of a stronger gel.

Thus, the improved renneting properties of milk ultrasonicated at pH 8.0 and
re-adjusted to 6.7, reported in this chapter, are due to the smaller size and altered structure of the ultrasound-induced newly-formed micelle particles.

7.4.2 Viscosity of reconstituted skim milk during concentration

Normally, to study the viscosity of milk during concentration, an in-line viscometer is used for an immediate viscosity measurement without extending the hold-up time before measurement, because the viscosity of the milk concentrate is positively correlated to the sample holding time (Bloore & Boag, 1981). Although the viscosity was measured in a rheometer in this study, the difference in the holding time of each sample was generally no more than 10 s, which gave a relatively reliable comparison between samples. The results showed that the ultrasonicated milk that was concentrated to > 40 % TS had a higher viscosity compared to untreated milk at the same concentration. Apart from the processing conditions such as temperature or sample holding time, the viscosity of milk concentrate is largely dependent on the voluminosity of the macromolecular components, which are mainly caseins and whey proteins (Snoeren, Damman, & Klok, 1982). The voluminosity of these proteins in milk is determined by the protein composition and the pre-heat-treatment of milk (Snoeren, et al., 1982). In addition, Walstra (1979) discussed the voluminosity of the casein micelles based on different models of micelles and one of the factors that affects the voluminosity of casein micelles is micelle size. As caseins constitute about
79% of the proteins in milk and are mostly (~95%) present in micelles, any change in the physicochemical properties of casein micelles (e.g. size, structure) will affect the voluminosity, which in turn will have a large effect on the viscosity of the milk concentrate. Therefore, the increase in viscosity of the ultrasonicated treated milk during concentration compared to that in untreated milk again indicates the newly-formed ultrasound-induced micelle particles have smaller size and different structure compared to the original micelle particles.

### 7.4.3 Effects of US on the GDL-induced gelation properties of heated milk

Table 7.4 shows that heated milks had much lower gelation time, higher gelation pH and formed much stronger gels compared to untreated milks at all pHs. These results are in line with previous studies (Lucey & Singh, 1997; Lucey et al., 1997 & 1998). After the application of US processing to heated milks, the gelation properties of HUS milks were not appreciably different from those of heated milks at corresponding pH (Figures 7.7 & 7.8, Table 7.4).

The GDL-induced gelation of milk is largely dependent on the heat-induced whey protein aggregates in milk. Various studies have shown that the yoghurt gel firmness (G’) was positively related to the amount of denatured whey proteins (Dannenberg & Kessler, 1988b; McKenna & Anema, 1993). Furthermore, Anem, Lee, Lowe, and
Klostermeyer (2004) clearly demonstrated that the distribution of the heat-induced whey protein aggregates was also an important factor in determining the $G'$. Their results showed that in the pH range of 6.5 to 7.1, the final $G'$ was higher, gelation time was shorter and gelation pH was higher in the milks that had been heated (90 °C/30 min, where the native whey proteins were close to fully denatured in milks at all studied pHs) at higher pH. The results of current study are in line with those of Anema, Lowe, and Lee (2004) with respect to milks heated at pH 6.3 and re-adjusted to 6.7 and milks heated at pH 6.7 (Figure 7.7). However, there was no noticeable difference in gelation properties between heated milk at pH 6.7 and heated milk at pH 7.1 and re-adjusted to 6.7. In the study by Anema, Lowe, and Lee (2004), a positive relationship between the amount of soluble denatured whey protein and the final $G'$ was observed, but there was no relationship between the amount of micelle-bound denatured whey proteins and the final $G'$. This indicated that it is the heat-induced protein aggregates in the serum that have a significant effect on the gel firmness. These authors attributed this to the denatured whey proteins in the serum phase increasing the number of aggregating particles during gelation, which consequently increased the contacting points and interconnectivity between particles and led to a firmer gel (Anema, Lowe, & Lee, 2004). In our study, US processing clearly increased the heat-induced whey protein aggregates in the serum phase (Chapter 6, Section 6.3.3, Figure 6.6), particularly at pH 6.7. Moreover, the particle size of these serum aggregates and casein micelles was decreased significantly (Chapter 6, Section 6.3.3, Figure 6.7 a), which in turn increased the total surface area of the
particles for aggregation. Therefore, it could be expected that there are more particles with higher total surface area in the HUS milks promoting gelation and formation of stronger gels. However, the results presented in Table 7.4 show that US processing did not improve the gelation properties of heated milks. On the other hand, the results in our study are in agreement with other studies (Alexander & Dalgleish, 2005; Donato, Alexander, & Dalgleish, 2007) which suggested that the primary factor affecting acid-gelation behaviour is the total amount of heat-induced aggregates formed in milk. The heat-induced aggregates in the serum phase associate with the surface of the casein micelles at the early stage during acidification before the gelation starts, turning the serum aggregates into micelle-bound aggregates. Thus, the distribution of the heat-induced aggregates between micellar and serum phases has little effect on the gelation behaviour.

7.4.4 Heat stability of recombined concentrated milk

Due to the seasonal effect on the heat stability of milk (Holt, Muir, & Sweetsur, 1978), the heat stable milk powder production in Australia is limited to the period from October to March (Augustin, Clarke, & Greenwood, 1990). Augustin et al. (1990) have shown that high-heat treatment (85 °C/30 min) of milk in late autumn and winter (May to June) alone was not enough to acquire heat stability for manufacturing recombined concentrated milk, but the addition of disodium hydrogen phosphate in pre-heated (85 °C/30 min) milk can acquire adequate heat stability.
In current study, the base skim milk powder was manufactured in June, which is outside the heat stable milk season. The application of ultrasound processing to pre-heat milk (90 °C/10 min) in this study did not improve the heat stability, despite the US-induced changes in the particle size and composition of the soluble aggregates (Chapter 6, Section 6.3.3). It is known that addition of phosphate, citrate (Augustin & Clarke, 1990) or removal of colloidal calcium phosphate (Fox & Hoynes, 1975 b) in milk can increase the heat stability as a result of the reduced Ca activity. In Chapter 6 (Section 6.3.2.2, Figure 6.5 c), it was shown that ultrasound processing did not change the inorganic phosphate content in heated milk serum (Chapter 6, Section 6.3.2.2, Figure 6.5 c) which suggests that the calcium phosphate equilibrium between the micellar and serum phase is not disturbed by ultrasound processing. Thus the Ca activity in heated milks was most likely unchanged after the US processing. Results of this study show that the modification of the protein particles in high-heat treated milk by ultrasound processing does not improve the heat stability of recombined concentrated milk. It appears that milk salts, particularly calcium and phosphate, may be the predominant governing factor in terms of heat stability, rather than the physical and chemical properties of heat-induced serum aggregates.
7.5 Conclusion

Ultrasound processing (20 kHz) of milk at pH 8.0 followed by pH adjustment back to natural milk pH 6.7 improves the rennet-induced gelation properties of milk and changes the viscosity of milk during concentration. These results provide valuable information about ultrasound applications in milk processing in the dairy industry. In contrast, ultrasound processing of heated milk did not have significant effects on the GDL-induced gelation properties and heat stability of recombined concentrated milk, despite the observed changes in particle size and protein partitioning described in this chapter and in Chapter 6.
Chapter 8

Final conclusions and future directions

8.1 Final conclusions

The dairy industry has always been at the forefront of trialling novel processing technologies. Both pulsed electric fields and ultrasound processing technologies have shown the potential to modify the physicochemical and functional properties of milk proteins. In this study, both processing technologies were investigated with the objective to study the ability of these processes to modify the proteins in reconstituted skim milk, with the particular emphasis on the casein micelles. The modified milks were then selected for further functional tests including renneting properties, viscosity, GDL-induced acid gelation and heat stability, which are closely related to the production of cheese, milk powder, yoghurt and evaporated condensed milk in the dairy industry.

PEF processing

In this study, it is concluded that under the processing conditions tested, PEF per se does not induce whey protein denaturation or casein micelle disruption in milk.

PEF processing technology is designed with the main purpose of inactivating
microorganisms while retaining food’s natural attributes. In this study, the PEF processing conditions, including E-strength (up to ~ 55 kV/cm), specific energy (up to ~ 500 kJ/kg) and processing temperature (maximum temperature ~ 70 °C), were generally higher than those used for microbial-inactivation purposes in most of the previous studies on milk (Sampedro, Rodrigo, Martinez, Rodrigo, & Barbosa-Cánovas, 2005). In addition in my PhD studies, the stability of the casein micelles was deliberately reduced to a large extent, by increasing the electrostatic repulsion between the casein molecules by increasing the pH of the milk or by dissolving the micellar calcium phosphate with the addition of a calcium chelating agent EDTA. However, even under these extreme conditions, PEF per se did not change the particle size or the surface charge of the casein micelles. The dissociation state of casein micelles and the phosphate distribution between the serum and the micellar phases were also not altered, neither was there whey proteins denaturation. Therefore, even when higher energy needs to be used in PEF for inactivation of microorganisms, the milk preserved by PEF is likely to retain its natural characteristics for further functional uses due to the lack of the effect of PEF on the milk proteins.

**Ultrasound processing**

In this study, it is concluded that US processing (particularly at 20 kHz), which disrupts casein micelles and creates micelle-like particles with smaller size in milk; US processing (20 kHz), which disrupts the protein particles and changes the distribution of the proteins between the micellar and serum phases in
high-heat treated milk, lead to:

(a) significant changes in renneting properties, as demonstrated by the decrease in rennet-induced gelation time, increase in rennet curd firmness and connectivity in the gel network and

(b) changes in viscosity during concentration, where there is an increase in viscosity at concentration between ~ 40 % and 55 % TS.

(c) no change in GDL-induced acid gelation properties (gelation time, gelation pH and gel strength) and the heat stability.

In Chapter 5, it was demonstrated that US effects on the casein micelles at 20 kHz were greater than those at 400 kHz under the same specific energy. The US effects on the casein micelles at 1600 kHz were negligible. Therefore the physical effects associated with and generated by low frequency US processing are the main contributors to the disruption of casein micelles in milk. In addition, this disruption was more marked in milk at higher pH (in the pH range 6.7 to 8.0), which indicated that increasing the electrostatic repulsion between the casein molecules can lead to greater US-induced disruption of casein micelles in milk. The disruption of the casein micelles led to the formation of small (~ 43 nm) micelle-like particles in the serum phase. To translate the effects of these US-induced changes in the milk proteins to the technological functions of milk, selected US treated milks were tested for renneting properties and for viscosity during concentration as described in Chapter 7. It was
shown that the renneting properties (renneting time, rennet curd firmness and rennet curd network) in milk that was ultrasonicated at pH 8.0 were significantly changed, compared to those in non-US milks or milk ultrasonicated at pH 6.7. These altered renneting properties may have benefits in cheese-making such as decreasing the time to produce cheese curd or increasing the firmness of the cheese curd. Additionally, milk ultrasonicated at pH 8.0 showed higher viscosity at the concentration higher than 40 % total solids, compared to non-US milk. This US-induced functional property of milk may have the potential for novel dairy product developments.

The protein system in high-heat treated milk is distinctly different from that in non-heated milk and is of great technological interest in the dairy industry, particularly in yoghurt-making or processes requiring heat stability. In Chapter 6, it was shown that US processing at 20 kHz could disrupt the casein micelles and the heat-induced soluble aggregates in high-heat treated milk. US processing also altered the distribution of the proteins between the micellar and serum phases by detaching the heat-induced aggregates on the micelle surface into the serum. Marked US-induced effects were observed in high-heat treated milk at natural pH 6.7. Despite these changes, when GDL was added into this milk to produce an acid gel, the gelation time, gelation pH and the gel strength did not significantly change, compared to those of non-US high-heat treated milk. This may be due to the GDL-induced gelation process being dominated by the amount of heat-induced aggregates rather than the distribution and size of these aggregates. In addition, the heat stability of the
recombined concentrated milk made from high-heat treated milk ultrasonicated at pH 6.7, is not improved, compared to that made from non-US high-heat treated milk.

8.2 Future directions

US processing under the tested conditions modifies the casein micelle structure leading to the changes in selected functionalities of milk. In order to realise the potential of this emerging technology and explore its industrial applications, and to fully elucidate the mechanisms of its effects on milk proteins, the following research work should be carried out:

- The milk used in this study was reconstituted from low-heat skim milk powder. It is worthwhile to conduct ultrasound processing experiments on raw milk to explore the commercial opportunity of ultrasound in dairy processing.

- Investigate other factors that are affecting micelle stability in US processing in milk. For example, In Chapter 5, we have shown that the electrostatic interaction between the casein molecules has marked effects on the US-induced disruption of casein micelles. Besides electrostatic interactions, other factors that can influence casein micelle structure such as content of MCP in micelles, hydrophobic/hydrogen interactions between casein molecules, or altered concentration of κ-caseins. In order to have a better understanding of the
mechanism of US-induced disruption of the casein micelles, US processing should be applied to milks which a) vary in the amounts of MCP (achieved by adding different levels of a Ca-chelating agent such as EDTA), b) vary in the strength of hydrophobic/hydrogen interactions (achieved by adding different levels of urea or sodium dodecyl sulfate), or c) vary in the concentration of surface κ-caseins (achieved by adding different levels of rennet to produce casein particles with altered surface coverage of κ-casein).

- Investigate the chemical effects of US processing on the US-induced disruption of casein micelles. Madadlou et al. (2009b & 2012) claimed that the US-induced disruption of micelle particles was more effective at 130 kHz compared to that at 35 kHz, due to greater chemical effects at the higher frequency. In my PhD studies, it has been shown that the chemical effects (i.e. the amount of radicals generated in US processing) were higher at 400 kHz compared to that at 20 kHz (Chapter 3, Figure 3.6). In addition, it was demonstrated that the US-induced disruption of casein micelles was more effective at 20 kHz than 400 kHz (Chapter 5). Our results appear to contradict those of Madadlou et al. (2009b & 2012). However, neither of the studies by Madadlou et al. (2009b & 2012) nor the studies in this thesis has showed direct evidence of the relationship between US-induced disruption of casein micelles and the chemical effects generated by US processing. To investigate the chemical effects on casein micelles in milk during US processing, the chemical forces must be separated
from the physical forces. The amount of radicals generated during US processing under a certain processing specific energy can be determined as described in Section 3.5.2.5, Chapter 3. Accordingly, certain amount of chemicals that can generate equal amount of radicals as determined previously in the US processing is added into milk. Analysis is then carried out to examine the effects of these radicals on casein micelles. The chemical effects on casein micelles can therefore be determined.

• In Chapter 7, the heat stability of recombined concentrated milk made from ultrasonicated high-heat treated milk was tested. However, the heat stability of all the tested samples was not improved despite the US-induced modification of the protein system as shown in Chapter 6. It is known that the heat stability is highly dependent on the season when the milk is produced. The interaction between the US processing and the seasonal effects on heat stability may provide important information to explore the application of US processing in the dairy industry. Therefore, the effects of US processing in the heat stability of recombined concentrated milk should be carried out from milk that is produced at several different seasons of the year.

• In Chapter 7, it was reported that milk ultrasonicated at pH 8.0 showed superior renneting properties. It may be worthwhile to carry out the investigation of the US-modified milk functionality across the whole cheese-making process and
investigate the interaction between the US-modified milk proteins and the acidification from the starter culture, the coagulation of the milk, the heating process of the raw cheese curd and the maturation of the cheese.
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