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Aggregation and Gelation Characteristics of High Protein Dairy Ingredient Powders

Thesis presented by

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for the degree of

Doctor of Philosophy

in

Food Science and Technology

Dec 2017

Declaration

I hereby declare that the work submitted is entirely my own and has not been submitted to any other university or higher education institute, or for any other academic award in this university.

Signature:

_____ Date _____

Yingchen Lin

This thesis is dedicated to my parents

with love, respect and gratitude

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Abstract

Milk protein-containing dairy ingredient powders are used extensively in the formulation of a wide range of foods and beverages. Protein is an essential component in such food systems, where it provides nutrition and a range of functionalities including rennet gelation, heat stability, ethanol stability and acid gelation, which influence the manufacture and stabilization of the foods. The protein dispersed in water is typically subjected to various processes, including concentration, homogenization, heating, acidification, in the presence of other formulation constituents (e.g., salts, sugars, ethanol, and acids) during food processing. The exposure of proteins to different processing steps and other constituents can significantly alter their aggregation behaviour, and hence, stability. The current study investigated the changes in compositional, physico-chemical and functional properties of skim milk and protein-based dispersions such as reconstituted skim milk, protein-fortified milk, and reconstituted milk protein concentrate, which resulted from seasonal variations, fortification of milk with different high protein ingredients, processing operations (heat treatment, evaporation and spray-drying) during the manufacture of skim milk powder, at pH during milk heat treatment followed by restoration of pH after heat treatment. Seasonal variations in proportion of spring- and autumn-calving milk in mixed-herd milk and in milk composition were characterized. Key factors influencing the functionality of the milk protein included the calcium-phosphate content, heat treatment of milk during powder manufacture, pH adjustment prior to heat treatment, and the composition of the solvent. These factors exerted their effects by altering one or more physico-chemical parameters of the reconstituted ingredients, including denaturation of whey

protein, complexation of denatured whey proteins with dissociated κ-casein or casein micelles, partitioning of individual caseins and minerals between the serum and the casein micelle, casein micelle size and charge.

The results of the current studies have expanded our knowledge of the factors affecting key functional parameters of reconstituted milk protein powders, and provide mechanistic bases for understanding how these factors exert their effects.

List of publications

1. Lin, Y., Kelly, A.L., O'Mahony, J.A., and Guinee, T.P. (2016). Fortification of milk protein content with different dairy protein powders alters its compositional, rennet gelation, heat stability and ethanol stability characteristics. *International Dairy Journal, 61,* 220-227.
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2. Lin, Y., Kelly, A.L., O'Mahony, J.A., and Guinee, T.P. (2017). Addition of sodium caseinate to skim milk increases nonsedimentable casein and causes significant changes in rennet-induced gelation, heat stability, and ethanol stability. *Journal of Dairy Science, 100,* 908-918.
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3. Lin, Y., O'Mahony, J.A., Kelly, A.L., and Guinee, T.P. (2017). Seasonal variation in the composition and processing characteristics of herd milk with varying proportions of milk from spring-calving and autumn-calving cows. *Journal of Dairy Research, 84,* 444-452.
   (Based on Chapter 2)

4. Lin, Y., Kelly, A.L., O'Mahony, J.A., and Guinee, T.P. (2017). Altering the physico-chemical and processing characteristics of high heat treated skim milk by increasing the pH prior to heating and restoring after heating. *Food Chemistry, 245,* 1079-1086.
   (Based on Chapter 6)

5. Lin, Y., Kelly, A.L., O'Mahony, J.A., and Guinee, T.P. (2018). Effect of heat treatment, evaporation and spray drying during skim milk powder manufacture on the compositional and processing characteristics of reconstituted skim milk and concentrate. *International Dairy Journal, 78,* 53-64.
   (Based on Chapter 5)

Chapter 1

Literature review

1. Milk

Global milk production has steadily increased from 790 to 819 million tonnes from 2014 to 2016, of which 82% is cow milk (Griffin, 2017). The term 'milk' in this thesis refers to cow milk unless otherwise stated. The main biological function of milk is to provide the neonate with complete nutritional requirements. As well as milk for direct consumption as pasteurised, sterilised or ultra-heat treated (UHT) milk, milk is further processed to dairy products and ingredients such as cheese, butter, condensed milk, and yoghurt and a range of milk powders. These applications require functional properties including rennet coagulability, heat stability, ethanol stability (ES) and acid coagulation, which are affected by the composition and processing conditions applied to milk.

Milk comprises water, lactose, fat, protein and ash (O'Mahony & Fox, 2013). Of particular importance to the physico-chemical and functional properties of skim milk are the protein and minerals.

1.1. Milk Protein

Protein constitutes ~3.5% (w/w) of milk, which is composed of two major groups, caseins and whey proteins, at a weight ratio of 8:2 (O'Mahony & Fox, 2013). In addition to casein and whey protein, non-protein nitrogen (NPN), fractionated in 12% trichloroacetic acid (TCA) solution, also exists in milk at a level of ~5% of total nitrogen; ~50% of NPN is urea (Muir & Sweetsur, 1976; O'Mahony & Fox, 2013).

1.1.1. Casein

Casein, constituting 2.8% (w/w) in milk or 75-80% of total protein, comprises of four main types, namely α_{s1}-, α_{s2}-, β- and κ-casein, in the weight ratio 4:1:3.5:1 (Huppertz, 2013). Casein can be fractionated from milk by adjusting the

pH to 4.6. Alternatively, ultracentrifugation can be employed to separate casein micelles with little loss in the level of colloidal calcium phosphate (CCP; O'Mahony & Fox, 2013). The resultant casein pellet generally maintains the properties of native casein micelles, and can be re-suspended in an appropriate medium to form a serum protein-free system (O'Mahony & Fox, 2013). However, dissociation of small amounts of casein (~10%) can occur on ultracentrifugation at 20°C (Dalgleish & Law, 1988). Some compositional and physico-chemical properties of individual caseins (α_{s1}-, α_{s2}-, β- and κ-casein) are shown in Table 1.1. β-Casein contains a high level of proline residues, coinciding with low levels of α-helix or β-sheet structures. Disulphide bonds are present in α_{s2}- and κ-casein, which facilitate the occurrence of sulfhydryl disulphide bonds interchange reactions (SH/S-S) between κ-casein and denatured β-lactoglobulin (β-Lg) when milk is heated. α_{s2}-Casein is the most hydrophilic of all caseins, whereas β-casein is the most hydrophobic, because of the higher content of phosphorylated residues in the former than the latter (one phosphorylated residues). κ-Casein is characterised by negative charge and is amphipathic containing both a hydrophobic region at the N-terminal and a hydrophilic region at the C-terminal, which is glycosylated. The negatively-charged hydrophilic region of κ-casein is of particular relevance to the stability of casein micelle. α_{s1}-, α_{s2}- and β-Caseins are sensitive to calcium ions ($[Ca^{2+}]$) and tend to aggregate in the presence of $[Ca^{2+}]$, owing to the existence of phosphorylated groups and phosphate centres. Other than phosphorylated groups, carboxyl groups of glutamic acid and aspartic acid residue are also able to bind $[Ca^{2+}]$ (Bingham et al., 1972). In contrast, κ-casein is not precipitated by $[Ca^{2+}]$ and is able to interact with the Ca-sensitive caseins *via* the hydrophobic region (Horne, 2002).

1.1.2. Casein micelle structure

With the aid of κ-casein, the casein micelle is stabilised through cross-linking of CCP, which causes dissolution of casein micelles if chelated by ethylenediaminetetraacetic acid (EDTA) (Lin et al., 1972). The stability of casein micelles in milk is critical for the biological function of milk, which transports Ca, P and protein from the mammary gland to the neonate. CCP is the dominant factor that maintains the integrity of the casein micelle, but other factors also contribute, most noticeably the non-covalent forces such as hydrogen bonding, electrostatic interactions, van der Waals, and hydrophobic interactions (de Kruif et al., 2012).

Table 1.1. Compositional and physico-chemical characteristics of milk proteins (Dalgleish & Law, 1988; Kinsella et al., 1989).

	Caseins (main)				Whey proteins (main)	
Items	α_{s1}-	α_{s2}-	β-	κ-	α-Lac	β-Lg
Partition of proteins in milk at 20°C						
Micellar phase (%, w/w)	1.09	0.3	0.9	0.29	n/a[1]	n/a
Serum phase (%, w/w)	0.07	0.01	0.13	0.05	n/a	n/a
Isoelectric point (pI)	4.96	5.27	5.2	5.54	4.2-4.5	5.2
Molecular weight (kDa)	23.6	25.5	24.0	19.0	14.2	18.4
Number of						
Total amino acid residuals	199	207	209	169	123	162
Phosphorylated residues	8-9	10-13	5	1	0	0
Phosphate centre	2	3	1	0	0	0
Proline residues	17	10	35	20	8	2
Disulphide bonds	0	1	0	1	4	2
Free thiol group	0	0	0	0	0	1
Sensitive to Ca	yes	yes	yes	no	no	no
Sensitive to Chymosin	no	no	no	yes	no	no

[1]n/a represents not applicable

Several models of casein micelle structure have been proposed in the literature and it still a topic of debate. Different models of substructure of the casein micelle have been proposed including the submicellar model (Schmidt, 1982), a dual-binding model (Horne, 1998) and the most accepted open rheomorphic structure (Holt, 1992). Despite no consensus on a defined substructure of the casein micelles, some features of casein micelles are generally agreed: (i) casein micelles bind considerable water, e.g., 2.5-4.0 g water/g protein (Sood, Gaind, & Dewan, 1979); (ii) the internal-located α_{s1}- and α_{s2}-caseins in the micelles, and the partially buried β-casein in the interior of the micelles, allowing hydrophobic interaction and binding with other casein molecules; (iii) caseins are associated through Ca bridges or CCP; (iv) casein micelles are stabilized in milk due to the presence of hydrophilic C-terminal at the end of κ-casein, protruding into the serum from the surface of the casein micelle and forming a salted polyelectrolyte hairy layer (de Kruif & Zhulina, 1996). The hairy layer of κ-casein provides stability to casein micelles by both steric and electrostatic stabilization, with the former being predominant; (v) casein micelles are considered spheroid and their size can range from 100 to 250 nm (Glantz, Devold, Vegarud, Lindmark Månsson, Stålhammar, & Paulsson, 2010).

pH and temperature are of particular importance to the stability of casein micelle structure. Increasing pH of milk to ≥7.5, results in dissociation of casein and increases in casein micelle size (Sinaga et al., 2017). Simultaneously, the milk becomes transparent by light-scattering of intact casein micelles. Decreasing pH of milk increases the solubilisation of CCP, which is evidenced by increases in the level of Ca and inorganic P in the serum (soluble) phase (Dalgleish & Law, 1989). The solubilisation of CCP is a temperature-dependent process, and increases as temperature decreases at pH >6.0 (Davies & White, 1960). At pH ~5.0, virtually all

the CCP dissolves in the temperature range 4 to 30°C (Dalgleish & Law, 1989). The pronounced solubilisation of CCP is accompanied by dissociation of caseins in the pH range 5.5-5.0. The dissociation of individual casein is also temperature-dependent; at 4°C, the proportion of soluble casein is higher than at 30°C. The dissociation rate of individual caseins is of the order κ-casein > β-casein > α_s-casein (Dalgleish & Law, 1988). Therefore, it is important to overcome cold-aging prior to analysis if milk has been kept at low temperatures for a long time.

1.1.3. Whey proteins

In contrast to casein, whey proteins are soluble at pH 4.6 in milk. Whey proteins are a heterogeneous group consisting of α-lactalbumin (α-Lac), β-Lg, bovine serum albumin, immunoglobulins, minor proteins and enzymes (Dupont et al., 2013; Havea et al., 2001). The major types of whey proteins α-Lac and β-Lg represent ~20 and 50% of total whey protein, respectively. Both α-Lac and β-Lg are hydrophobic globular proteins, characterized by a high level of secondary and tertiary structure. Whey proteins are generally more heat-labile than caseins, due to their complexity in structure.

α-Lac, representing 0.12% in milk, has low molecular mass, ~14.2 kDa, and comprises 123 amino acids, with four disulphide bonds (Karman & Van Boekel, 1986). The absence of a free thiol group prevents it from interacting directly with κ-casein on heating (Table 1.1). α-Lac is capable of binding Ca, which connects the α-helical and β-sheet domains in the native α-Lac. It can also bind zinc and other metals, and is therefore known as a metalloprotein. β-Lg exits as a globular negatively-charged dimer at a concentration of 0.32% in milk (Karman & Van Boekel, 1986). β-Lg contains two disulphide group and one free thiol bond which is

exposed on heating (>60°C), allowing β-Lg to react with κ-casein and/or α-Lac *via* SH/S-S interchange reaction mechanisms.

1.2 Minerals in milk

The minerals of milk constitute 0.8-0.9% (w/w) of milk, and include calcium, magnesium, sodium and potassium as the main cations and inorganic phosphate, citrate, sulphate and chloride as the main anions. At the native pH of milk, the ionic strength of milk is ~80 mM (Gaucheron, 2005). The minerals in serum (soluble) refer to those remaining non-sedimentable on ultracentrifugation while the minerals associated with casein micelles, and sedimented on ultracentrifugation, are denoted as micellar (non-sedimentable) minerals. Fat and lactose are not involved in binding of minerals (Gaucheron, 2005). The partition and form of minerals are given in Table 1.2 (Fox et al., 2015a).

The concentrations of Ca and P are 120 and 75 mg/100 g, respectively, of which 66% and 50% are micellar-bound Ca and P, respectively. A portion of micellar Ca can interact with colloidal inorganic phosphate to form CCP, which is important for micelle integrity. Other than that, the rest of micellar Ca is bound directly to casein by the intermediate of phosphoserine, known as organic phosphate. Therefore, micellar Ca include CCP and calcium caseinate, which involves inorganic phosphate and organic phosphate, respectively. However, the two types of micellar Ca may not be separable using ultracentrifugation (Gaucheron, 2005; White & Davies, 1958). About 1/6 of Ca (5 mM) is bound to organic P, which plays a critical role in the formation of acid gel (Famelart et al., 2009; Mekmene et al., 2009). A limited amount of Ca is also bound to α-Lac at a ratio of one Ca atom per protein.

Mg and citrate are also included in the micellar system and form part of the CCP structure.

Table 1.2. Partition of organic and inorganic salts between micellar and serum phases and their form in serum phase of milk. Table adopted from Fox et al. (2015a).

	Concentration (mg/100 g)	Micellar (% total)	Serum (% total)	Form of serum minerals
Calcium (Ca)	120	66	34	35% Ca^{2+}
				55% bound to citrate
				10% bound to phosphate
Phosphate (P)	75	57	42	10% bound to Ca and Mg
				51% $H_2PO_4^-$
				39% HPO_4^{2-}
Citrate (Cit)	175		94	85% bound to Ca and Mg
				14% $Citr^{3-}$
				1% $HCitr^{2-}$
Magnesium (Mg)	13	33	67	35% Mg^{2+}
				55% bound to citrate
				10% bound to phosphate
Potassium (K)	145	8	92	Completely ionized
Sodium (Na)	50-60	8	92	Completely ionized
Chloride (Cl)	120	-[1]	100	Completely ionized
Sulphate (SO_4^{2-})	10	-	100	Completely ionized

[1]- represents not measurable.

2. Heat treatment of milk

Heat treatment of milk is used widely during milk processing and dairy product manufacture; with the aims of (i) reducing microbial risk by inactiviating temperature-sensitive microorganisms, especially pathogens; (ii) inactivation indigenous enzymes (such as lipoprotein lipase); (iii) improving texture of final dairy products (e.g., yogurt manufacture); and (iv) meeting processing operation

requirements (e.g., preheating of unconcentrated milk to improve heat stability of concentrated milk, preheating of milk to 30-35°C for cheese manufacture and to 40-45°C for starter culture fermentation in yogurt manufacture, warming of milk for UF processing operating at 50°C).

According to the time and temperature conditions, heat treatment can be categorised as thermization, pasteurization, forewarming and sterilization as summarised in Table 1.3.

Table 1.3. Categorise of heat treatment of milk and the corresponding time/temperature combination. Data adopted from Bylund (2003) and Fox et al. (2015b).

Process	Temperature (°C)	Time
Thermization	63-65	15 s
Pasteurization		
Low temperature × long time	63	30 min
High temperature × short time	72	15 s
Ultra high temperature × short time	125-138	2-4 s
Forewarming for sterilization	90-120	2-10 min
Sterilization		
Ultra-heat treatment	130-140	3-5 s
In-container sterilization	110-120	10-30 min

2.1. Heat-induced changes in milk

Heat treatment of milk affects pH and induces changes in the partition of κ-casein in milk, denatured whey proteins and minerals between serum and micellar phases, which are strongly associated with physico-chemical and functional properties of milk. Other components in milk are also influenced by heat treatment (e.g., vitamins and enzymes) are discussed elsewhere (Downs & Taylor, 2010; Ryley & Kajda, 1994).

2.1.1. pH

The reduction in pH on heating (from ~6.65-6.90 to ~6.18-6.30) was observed by carrying out *in situ* measurement of pH at temperature up to 90°C (Chandrapala et al., 2010; Chaplin & Lyster, 1988; Ma & Barbano, 2003). Chandrapala et al. (2010) demonstrated that on increasing the total solid of milk from 9 to 21%, the magnitude of the heat-induced decrease in pH increased by 0.1 pH units. Further study showed that the effect of heat treatment on pH was reversible after overnight holding at 25 °C, reflected by reversion in the pH in heated milk back to native pH (Chandrapala et al., 2010). However, if the milk was heated at pH ≥6.83, the pH of heated milk still remained low after equilibration. The difference between pH measured at high temperature and pH after equilibration increased as the pH prior to heat treatment was increased from 6.83 to 7.21 (Chandrapala et al., 2010). The reduction of milk pH after heating has been attributed to the thermal degradation of lactose to organic acids, such as formic acid, and the release of hydrogen ions from heat-induced precipitation of calcium phosphate, due to the decrease in solubility of calcium phosphate with increasing temperature (O'Connell & Fox, 2003; Rose & Tessier, 1959; van Boekel et al., 1989). Additionally, the precipitated phosphate could also be produced by heat-induced dephosphorylation of casein (Dalgleish et al., 1987).

2.1.2. Casein

No heat denaturation of casein is observed when milk is heated at temperatures from 70 to 100°C due to the random coil structure and lack of secondary and tertiary structure in casein (Law et al., 1994). Different from rennet-induced enzymatic hydrolysis (Section 3.1.1), heat treatment of milk results in dissociation of κ-casein and yields a Ca-sensitive/κ-casein-depleted casein micelle.

The instability of casein micelles can be due to the removal of κ-casein from the micelle or the collapse of the 'hairy layer' induced by acidification (de Kruif & Zhulina, 1996; Huppertz, 2016). The dissociation of casein is temperature-dependent on heating at pH ≥6.7. Anema and Klostermeyer (1997) reported that heat treatment increased proportions of casein, α_s- and β-casein in non-sedimentable (serum) phase, up to 70°C followed by a reduction on increasing temperature to 100 °C. At any given pH and temperature, the extent of casein dissociation follows the order: κ- > β- > α_s-casein (Anema & Klostermeyer, 1997). Casein micelle size in milk ranges from 100-250 nm (Anema et al., 2004; Glantz et al., 2010). Heat treatment of milk at natural pH leads to an increase in casein micelle size, which is attributed to the association of denatured whey protein with casein micelles. The increase in casein micelle size is determined by the severity of heat treatment, and hence the level of whey protein denaturation at native milk pH (Anema, 2007; Anema, 2009; Martin et al., 2007). Casein hydration is reduced on heating, possibly as a result of precipitation of calcium phosphate, the dissociation of κ-casein, and the association of denatured hydrophobic whey proteins with the casein micelles induced by heat treatment (Rüegg et al., 1979).

2.1.3. Whey proteins

α-Lac and β-Lg account for 20% and 50% of whey proteins, respectively. β-Lg is more heat-labile than α-Lac (Vasbinder et al., 2003). The level of whey protein denaturation increases as severity of heat treatment increases, which has been extensively studied (Anema & Li, 2003a,b; Carr, 1999; Guinee et al., 1997; Oldfield et al., 1998; Vasbinder et al., 2001).

On heating, the reactive thiol group at cysteine residue 121 and hydrophobic amino acids are exposed in heat-denatured unfolded non-globular β-Lg, which can form disulphide links with other reactive disulphide groups through thiol group-disulphide bridge interchange reactions (Croguennec et al., 2003). β-Lg can react with κ-casein through SH/S-S interchange reaction, thereby, becoming attached to casein micelles *via* covalent disulphide linkages. Apart from interactions with casein micelles, β-Lg can also associate with α-Lac, which contains four disulphide bonds. Further interactions that should be taken into account are the involvement of non-covalent interactions, including hydrophobic, electrostatic and van der Waal forces (Guyomarc'h et al., 2003; Oldfield et al., 2000). During heat treatment of milk, α-Lac does not attach to casein micelles due to the lack of free thiol groups unless through β-Lg acting as a bridge between α-Lac and casein micelles through thiol-disulphide interchange (Corredig & Dalgleish, 1996). The level of denatured whey protein precipitating on the casein micelle increases with increasing severity of heat treatment (Oldfield et al., 1998).

Heat treatment of milk at native pH results in mixture of native whey proteins, denatured whey protein aggregates in serum phase, denatured whey protein/ κ-casein complexes in the serum phase and denatured whey protein-coated casein micelle (Donato & Guyomarc'h, 2009; Guyomarc'h et al., 2003). The composition of the mixture is dependent on heating pH and temperature (Corredig & Dalgleish, 1996; Law et al., 1994; Oldfield et al., 2000). The effect of heating pH on the partition of κ-casein and denatured whey protein will be discussed in Section 2.2 in this chapter.

2.1.4. Minerals

Although heat-induced acidification may favour the solubilisation of calcium phosphate, it has been found that the reduction in solubility by heat treatment is the dominated result, evidenced by reduction in [Ca^{2+}] measured at high temperature (Chandrapala et al., 2010; On-Nom et al., 2010; Pouliot et al., 2009). Heat treatment of milk shifted calcium phosphate equilibrium between colloidal and serum phases, with a concomitant release of H^+ and reduction in milk pH, as shown by the following equation:

$$3Ca^{2+} + 2HPO_4^{2-} \underset{\text{Cooling}}{\overset{\text{Heating}}{\rightleftharpoons}} Ca_3(PO_4)_2 \downarrow + 2H^+$$

The extent of calcium phosphate precipitation increases with increasing heating temperature and time. The effect of heating on precipitation of calcium phosphate is considered as reversible on cooling if the heat treatment is not severe (e.g., 60-90°C for 10-20 min). The indigenous CCP may dissolve on cooling to partially restore the salt balance (Chandrapala et al., 2010; Rose & Tessier, 1959). Despite the restoration of mineral equilibrium, the newly released [Ca^{2+}] may be different from the original [Ca^{2+}], therefore the functional properties of heated milk are strongly altered (van Hooydonk et al., 1986). Following severe heat treatment (>100°C), this shift of calcium phosphate is irreversible (de la Fuente, 1998). The partitioning of other minerals including Na, citrate, K and Mg are not affected on heat treatment up to 104°C (Rose & Tessier, 1959).

2.1.5. Other components in milk

Lactose degrades on heating to galactose and organic acids, mainly formic acid. Glycation of proteins occurs during milk heat treatment by in conjugation of reducing sugars (e.g., lactose) with protein, resulting in condensation of a carbonyl

group (Ames, 1992), which significantly affects the functionalities of milk proteins such as foaming and gelation properties of milk proteins (Báez et al., 2013; O'Mahony et al., 2017; Spotti et al., 2013). Moreover, Maillard reactions, starting from the glycation of protein, occur in the presence of lysine (Berg & van Boekel, 1994; Huppertz, 2016; Turner et al., 1978), which leads to degradation of lactose to galactose and formic acid.

2.2 Effect of pH at heating on distribution of denatured whey proteins

Numerous studies have been conducted on the effect of heating pH on compositional changes in milk (Anema & Klostermeyer, 1997; Anema et al., 2011; Donato & Guyomarc'h, 2009; Guyomarc'h et al., 2003; Vasbinder et al., 2003; Vasbinder & de Kruif, 2003; Vasbinder et al., 2003). Heat treatment of milk at pH ≤6.7 leads to a considerable proportion of denatured whey protein (mainly β-Lg) interacting with κ-casein on the surface of the casein micelle *via* sulfhydryl/disulphide interchange reactions and hydrophobic reactions. Large amount of denatured whey protein located on the micelle surface and head-induced precipitation of Ca and P coincide with an increase in casein micelle size when heated at native pH or less than native pH of milk. The proportion of casein micelle associated denatured whey protein increased as pH at heating decreased (Anema, 2007; Anema & Li, 2003; Martin et al., 2007). On increasing heating pH to ≥6.8, κ-casein dissociates increasingly and complexes with denatured whey proteins in the serum phase (Anema et al., 2007; Ménard et al., 2005; Singh & Fox, 1985). The dissociation of κ-casein induced by heat treatment is concomitant with the reduction in casein micelle size (Anema, 2007). Following heat treatment at high pH (>6.7), the formation of κ-casein/ β-Lg complexes in serum was irreversible on reducing pH, as evident by the unchanged casein micelle size measured on acidification until to

the pH of acid gel onset (Anema et al., 2004). The changes in partitions of κ-casein and denatured whey protein markedly affect the functional properties of milk, e.g., acid gelation. However, information on the effect of restoration of heating pH to native milk pH after heating on physico-chemical characteristics and functionality of milk is not abundant.

3. Functional properties of milk

3.1. Rennet-induced gelation

In a series of processing steps for cheese manufacture (i.e., milk standardization, rennet coagulation, cooking, draining, pressing or modulation and ripening), rennet-induced gelation is one of the central steps during the cheese manufacture of rennet-curd cheese (e.g., Cheddar, Gouda and Mozzarella). A controlled rennet gelation is desired due to its significant influence on the composition, structure, texture and functionality of the final cheese products (Guinee, 2016).

3.1.1. Mechanism of rennet-induced gelation

Rennet-induced gelation of milk involves two consecutive stages, namely enzymatic (primary) and aggregation (secondary) stages (Corredig & Salvatore, 2016). The enzymatic stage is initiated when rennet is added to milk. Chymosin, one of the main milk-clotting enzymes in rennet, acts specifically at the bond between Phe_{105} and Met_{106} residues of κ-casein (Corredig & Salvatore, 2016). κ-Casein is located on the surface of casein micelle and provides stability of casein micelle by forming a polyelectrolyte layer creating steric and charge repulsion between the casein micelles. The hydrophilic C-terminal portion of κ-casein, referred to as glyco-

macro-peptide (GMP), having a hydrodynamic length of 7 nm (Corredig & Salvatore, 2016; Dalgleish, 1993; de Kruif, 1999), is hydrolysed from casein micelle, resulted in increased hydrophobicity of *para*-casein and reduction in overall charge of casein and stabilization by κ-casein (Corredig & Salvatore, 2016). The extent of hydrolysis of κ-casein is usually measured by addition of 2% or 12% TCA followed by determination of nitrogen level using Kjeldahl or reversed-phase high performance liquid chromatography (López-Fandiño et al., 1993; Wheelock, 1973; Wheelock & Penney, 1972). When 60-100% of κ-casein in skim milk is hydrolysed (Salvatore et al., 2011; Sandra et al., 2011), the density of the hairy layer and the steric stabilization effect decreases. The aggregation of *para*-casein occurs by knitting and fusion accompanied by the formation of rennet gel. The transition from liquid to rennet-gel can be monitored using low-amplitude strain oscillation rheometry which shows the formation of gel when the gel firmness, indicated by storage modulus (G'), is ≥0.2 Pa. Gel formation can also be detected using transmission diffusing wave spectroscopy (Sandra et al., 2012), which determines the changes occurring in the dynamics of the casein micelles during aggregation. The rennet gel formation is indicated by the increase in apparent diameter of casein micelles determined by diffusing wave spectroscopy.

3.1.2. Factors affecting rennet-induced gelation

Rennet coagulability is governed by protein, mineral and heat treatment of milk in the process of rennet gelation. Other determining factors including pH and temperature at renneting, concentration of rennet, homogenization and high pressure treatment, as have been extensively reviewed (Corredig & Salvatore, 2016; Nájera et al., 2003; Pandey et al., 2003; Saito, 1994; Sandra & Dalgleish, 2007; Zannoni et al., 1981).

3.1.2.1. Protein

As discussed above, casein is the essential material that forms a rennet-induced gel. Numerous previous studies have demonstrated the positive correlation between increasing protein content and rennet gelation properties of milk (Ferrer et al., 2008; Guinee et al., 1997; Guinee et al., 1996; Guinee et al., 2006; Jõudu et al., 2008; Nair & Corredig, 2015; Salvatore et al., 2011). It has been found that increasing protein content in skim milk or concentrated retentate resulted in markedly reduced in rennet gelation time, increase in gel firming rate, storage modulus (G') and cheese yield (Guinee et al., 1997; Guinee et al., 1996; Guinee et al., 1996; Jõudu et al., 2008). The changes are due to higher volume fraction of casein micelles and increases in collisions of calcium phosphate *para*-caseins (Guinee et al., 1997; Guinee, O'Callaghan et al., 1996; Guinee et al., 2006). G' increases more than proportionally with gel-forming protein content, as evidenced by a power law dependency of G' after a given duration of incubation with gel forming protein (P):

$G' \propto P^n$, where $2.0 < n < 2.6$ (Guinee, 2016; Guinee et al., 1996).

Serum casein, referred to casein remaining in the serum (soluble) phase under ultracentrifugation, has attracted considerable attention recently. The level of serum casein or whey protein/κ-casein complex can be increased by addition of sodium caseinate (Gaygadzhiev et al., 2011; Gaygadzhiev et al., 2012; Nair & Corredig, 2015) or heat treatment of milk (Kethireddipalli et al., 2010, 2011), respectively. The deterioration or complete inhibition in rennet gelation in milk with increased serum casein content is partially attributed to the impeded aggregation and knitting of *para*-casein due to the presence of soluble caseins. The presence of soluble caseins may prevent the *para*-caseins from aggregation by shielding the hydrophobic or calcium-

sensitive pathches on the surface of the caseins (Corredig & Salvatore, 2016). The increase in serum casein caused by addition of sodium caseinate (NaCas), or heat treatment of milk, did not, or to a very minor extent, affect the hydrolysis κ-casein during the enzymatic stage of rennet gelation (Anema et al., 2011; Gaygadzhiev et al., 2011; Gaygadzhiev et al., 2012; Kethireddipalli et al., 2010, 2011; Mollé et al., 2006; Nair & Corredig, 2015).

In addition to concentrations of casein and soluble casein, casein micelle size was found to be negatively correlated with rennet coagulation time and gel firmness (Ekstrand, Larsson-Raźnikiewicz, & Perlmann, 1980; Glantz et al., 2010). Smaller casein micelles contain more κ-casein (Donnelly et al., 1984), which favour rennet gelation.

Although native whey protein is not involved in the formation of rennet gels, Gamlath et al. (2018) found that the high ratio of native whey protein:casein (1:1 and 4:1) inhibited the hydrolysis of κ-casein in reconstituted skim milk, and hence delayed the rennet gelation process and reduced gel firmness. In contrast to increasing the ratio, reduction in native whey protein:casein ratio from 0.25:1 to 0.03:1 resulted in increases in gel firming rate and consequent gel strength. Gamlath et al. (2018) also suggested that the presence of a high level of native whey protein behaved as a physical barrier to aggregations *para*-casein.

3.1.2.2. The role of minerals in rennet gelation

Chymosin activity is affected by the presence of salts in milk serum in respect of ionic strength and type of ions. It has been reported that rate of κ-casein hydrolysis was increased or reduced by increasing ionic strength with addition of 8 mM $CaCl_2$ or NaCl, respectively (Bringe & Kinsella, 1986; Visser et al., 1980).

Grufferty and Fox (1985) reported the increase in rennet gelation time but no effect on the curd tension and syneresis rate of curd on addition of NaCl. The effect may be due to the competition between Na and Ca for sites on the *para*-casein micelles and exchange of Ca for Na, as seen by the increase in soluble calcium with increasing added NaCl. The rennet coagulation time of milk increases on addition of NaCl, but curd strength and the syneresis rate of rennet curd are not affected, as long as the increased rennet coagulation time is taken into account. The increase in rennet coagulation time following NaCl addition may be due to competition between Na^+ and Ca^{2+} for sites on the *para*-casein micelles (Grufferty & Fox, 1985).

Considering all ions in serum phase of milk, calcium ions are the most critical and essential ones for rennet-induced gelation of milk, as demonstrated in many studies (Bringe & Kinsella, 1986; Martin et al., 2010; Sandra et al., 2012). The positive effect of calcium ions is partially attributed to the charge-shielding effect, reduction in steric repulsion between *para*-casein micelles and strengthening short-range interactions of caseins (Sandra et al., 2012). It is generally recognized that the increase in serum calcium on addition of calcium salts improves rennet gelation properties, reflected as a decrease in rennet gelation time and increased gel firmness (Sandra et al., 2012; van Hooydonk et al., 1986). However, excessive levels of added Ca^{2+} results in adverse effects on gel firmness (Udabage et al., 2001).

Calcium present in the micellar phase is known to be a key factor that responsible for casein micelle integrity. A weak and flexible rennet gel can be expected to form in micellar Ca-depleted milk prepared by reducing pH, adding chelating salts (e.g., EDTA and citrate) to milk, or dialysis of milk against water. The internal structure of the rennet gel is weakened due to the changes in cross-

linking and forces such as hydrogen bonds, van der Waals interactions and calcium bridges, which stabilize the rennet gel (Choi et al., 2007).

3.1.2.3. Heat treatment

As discussed earlier (Section 2.1), heat treatment (≥60-70°C) of milk leads to changes in the level and distribution of denatured whey protein, shifts in salt equilibrium between micellar and serum phases, and potential alteration of casein micelles including dissociation of κ-casein or its association with denatured whey protein.

It has been shown that heat treatment of milk results in deterioration in rennet gelation properties. The adverse effect may be synergistic from several aspects; firstly, the rennet-induced gelation of heated milk may be stabilized by association of denatured whey proteins with casein micelles. Heat treatment of milk at pH less than the natural pH of milk (~6.7) results in precipitation of denatured whey proteins on the casein micelle. The proportion of denatured whey proteins in the micellar phase increases as pH at heating reduces (Vasbinder et al., 2003; Vasbinder & de Kruif, 2003). Heat treatment of milk at pH >6.7 leads to formation of κ-casein-whey protein aggregates in the serum phase. The level of serum-soluble aggregates increases with heating pH. On addition of rennet, the rate and level of κ-casein hydrolysis is not affected by heat treatment of milk (Mollé et al., 2006; Vasbinder et al., 2003). A tentative mechanism of the role of denatured whey protein during rennet gelation has been proposed (Anema et al., 2011). For milk heated at pH <6.7, denatured whey protein is attached to casein micelle, and therefore the casein micelles are partially stabilized by denatured whey protein, which delays the aggregation process of the whey-protein-modified *para*-casein. Weak gels will be

formed provided enough time is given. In contrast, at pH >6.7, the κ-casein-depleted casein micelles are unstable, and therefore tend to aggregate with other casein micelles and with the GMP-depleted-κ-casein/whey protein complex in the serum phase. These aggregated particles will be partially stabilized by the denatured whey protein from the GMP-depleted-κ-casein/whey protein complex, which results in slow aggregation and knitting of aggregated particles and weak gel. In addition, (Kethireddipalli et al., 2010) also reported that heat treatment of milk at alkaline pH may alter the structure of casein micelle, which was indicated by the lack of development of rennet gel in suspensions of heated casein micelles and unheated serum after dialysis against unheated milk. Moreover, concentrations of serum calcium phosphate and ionic calcium are reduced on heating (Chandrapala et al., 2010; Van Hooydonk et al., 1987). The equilibrium of Ca and P between colloidal and serum phases may be re-established after long holding times. However, van Hooydonk et al. (1987) indicated that the Ca and P originally present in the serum phase of unheated milk were different from the re-solubilized Ca and P in the serum phase after heating and equilibrating of milk.

3.2. Heat stability

Heat stability of milk is defined as the ability of milk to withstand heat treatment, and thus not result in visible flocculation or gelation in milk, or formation of protein aggregates which precipitate on centrifugation (Huppertz, 2016). The importance of heat stability is due to the heat treatment applied to milk and milk concentrate products for microbial-safety reason and enhancement of functional properties in dairy products, such as yoghurt.

3.2.1. Assessment of heat stability

Several methods have been developed for assessment of heat stability from different aspects based on the changes induced by heat by measuring the heat-induced coagulation time (Miller & Sommer, 1940), using Klarograph for viscosity measurement (De Wit et al., 1986), magnetic device (Foissy & Kneifel, 2009) or ultrasound spectroscopy at elevated temperature (Lehmann & Buckin, 2005). Objective and subjective methods for determination of heat stability have been developed by Davies and White (1966) and Miller and Sommer (1940), respectively. The objective method involves heat treatment of milk at a certain temperature for different duration of time, low-speed centrifugation of heated milk (e.g., 300-400 *g*) and determination of sedimentable nitrogen on centrifugation. The result of heat stability of milk is expressed as the nitrogen-depletion as a function of heating time. At the point of heat aggregation, an increase in sedimented nitrogen will be observed in the curve of nitrogen-depletion *vs.* heating time. The subjective method for determination of heat stability was developed by Miller and Sommer (1940) and modified by Davies and White (1966). The concept of heat coagulation time (HCT) was introduced and defined as a period time from samples subjected to high temperature to the occurrence of visual flocculation in samples. A temperature of 140°C is normally used for concentrated milk samples while 120°C is used for concentrated milk samples. In this method, a thermostatically controlled oil bath and gentle rocking of samples in oil bath during measurement are essential. Samples are usually assayed for HCT as a function of pH ranging from 6.2-7.2. The result of subjective method can be demonstrated using HCT/pH curve, which is shown in Figure 1.1. The HCT in the subjective method can be well-correlated to the time point at which a strong increase in sedimented nitrogen, which is observed in

objective method. Another subjective method, much less adopted compared to measuring the HCT of samples, involves determination of the heat coagulation temperature (HCTemp) at which the HCT is 2 min.

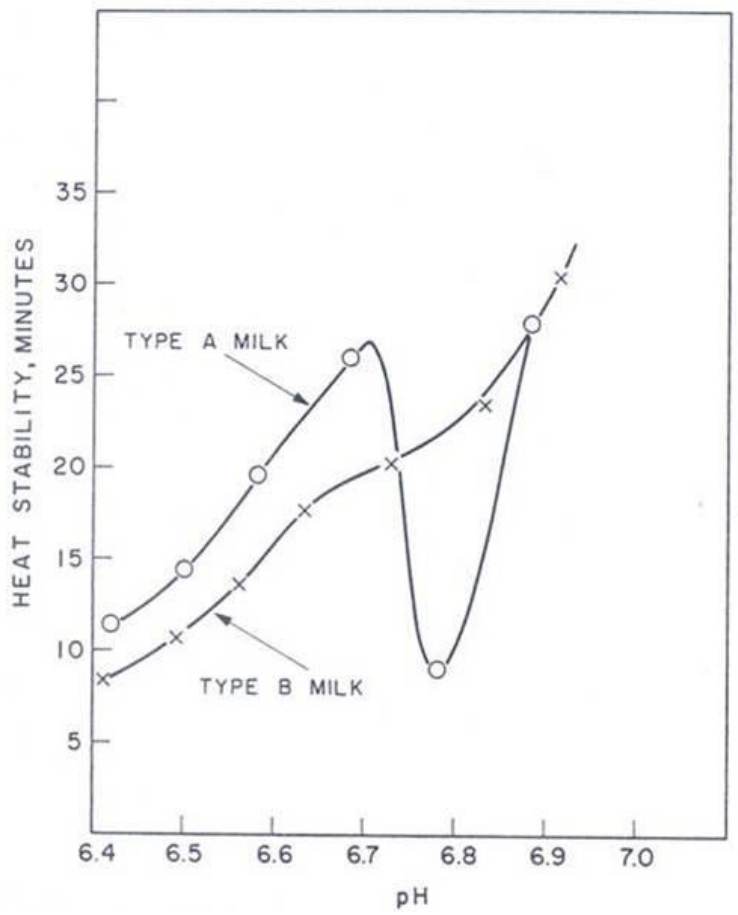

Figure 1.1. Example of heat coagulation time as a function of pH (HCT/pH profile) in type A and type B milk. Data adapted from Tessier and Rose (1964).

Subjective method is the most frequent experimental method in determining heat stability, which records the HCT and plots HCT/pH profile (Huppertz, 2016). Two types of HCT/pH profiles of unconcentrated skim milk (~9-11% total solids) are usually obtained, namely type A and type B. Type A HCT/pH profile shows increased HCT as a function of pH to maximum HCT (HCT_{max}) at pH 6.6-6.8, and decreased HCT at pH 6.9-7.0, resulting in a minimum HCT (HCT_{min}) prior to the increased HCT again as pH increases at pH ≥7.1. HCT increases continuously as a

function of increasing pH in type B HCT/pH profile (O'Connell & Fox, 2003). For concentrated milk, the HCT increases as pH increases to an HCT_{max} at which the pH is close to native pH of concentrated milk and then decreases as pH increases further.

3.2.2. Factors affecting heat coagulation time of skim milk

3.2.2.1. Physico-chemical and compositional factors affecting heat stability

According to the HCT/pH profile, the effect of pH on heat coagulation time of milk is largely dependent on the type of milk. The effect of pH is much simpler as the HCT increases with increasing pH in milk. In type A milk, the HCT behaved differently at different pH values. On increasing the pH of milk up to the HCT_{max} during the heat stability test, HCT is increased as a result of the reduction in $[Ca^{2+}]$ and the precipitation of denatured whey proteins on the surface of the casein micelle. The increase in zeta potential also contributes to the enhanced heat stability (Schmidt & Poll, 1986). The decrease in HCT on increasing pH from pH 6.8 to 7.0 at which the HCT_{min} occurs is attributed to the dissociation of κ-casein at pH >6.6-6.7 (native milk pH). The resultant κ-casein-depleted casein micelles are very susceptible to heat due to the high sensitivity of α_{s1}-, α_{s2}- and β-caseins to Ca^{2+}. The formed κ-casein/β-Lg complexes remain in the serum phase. The HCT increases as a function of pH at the alkaline side of the HCT_{min}, due to the increase in zeta potential and decrease in $[Ca^{2+}]$ (O'Connell & Fox, 2003).

Tessier and Rose (1964) reported that the addition of κ-casein to milk converted type A HCT/pH profile to type B as the added κ-casein did not penetrate the micelles, indicating the protective effect of excessive amounts of κ-casein remaining on the surface of micelle. Addition of other caseins (i.e., α_{s1}-, α_{s2}- and β-casein) did not affect HCT of milk. In contrast to κ-casein, introduction of β-Lg to

serum protein free casein micelle dispersion, which exhibits type B HCT/pH profile, induces both HCT_{max} and HCT_{min} to the type B HCT/pH profile.

Types and levels of minerals in serum are critical to the heat stability of milk. The role of Ca in both colloidal and serum phases was extensively studied (Eshpari et al., 2014; Fox & Hearn, 1978; Fox & Hoynes, 1975; Grufferty & Fox, 1985; Kaushik et al., 2015; Sievanen et al., 2008; Singh & Fox, 1987). Addition of $CaCl_2$ to milk has a negative effect on heat stability (Kaushik et al., 2015; Sievanen, Huppertz, Kelly, & Fox, 2008). A similar negative effect of soluble Ca on heat stability was observed by Rattray and Jelen (1996) who standardized protein content of milk using ultrafiltrate from acid whey, resulting in an increase in soluble Ca.

The heat stability increases on the addition of calcium chelating agents to the milk (Augustin & Clarke, 1990; de Kort et al., 2012; Mohammed & Fox, 1986). A possible explanation for the specific effect of added calcium chelating agents on the heat stability of bovine milk is to influence the pH of the milk systems, which is indirectly related to its influence on the nature of the CCP.

It is well recognized that the positive effect of urea on heat stability of milk, in particular at the pH region of HCT_{max}, and the alkaline side of the HCT_{min} of type A milk, and at overall pH range of type B milk (Muir & Sweetsur, 1976; Muir & Sweetsur, 1977; Sikand et al., 2010). Urea is also the main component of non-protein nitrogen, and accounts for 60% of all NPN in milk. A positive correlation between NPN (in reality urea) and HCT_{max} was established in a study investigating the HCT and composition in bulk milk (Muir & Sweetsur, 1976; Muir & Sweetsur, 1977). When milk is heated at 140°C for a heat stability test, the urea in milk decompose gradually to ammonia and carbon dioxide (CO_2) (Metwalli & van Boekel, 1996).

The release of H^+, induced by precipitation of serum Ca, degradation of lactose to formic acid and thermal oxidation of reductones organic acids, is neutralized by ammonia or inhibited by CO_2 produced on urea decomposition (Metwalli & van Boekel, 1996). Therefore, the rates of decrease in pH and the heat-induced protein aggregation are reduced. A higher level of heat-induced dissociation of κ-casein and a reduction in ionic calcium were observed by Metwalli and van Boekel (1996). Another effect of urea degradation is the production of isothocyanate. Homocitrulline, formed by reaction of lysine and isothiocyanate, may enhance heat stability by modification of arginine with dicarbonyls or the further reaction with lactose resulting in reductions (Shalabi & Fox, 1982) in the Maillard reaction in heated milk and covalent polymerization of proteins (Metwalli & Van Boekel, 1996). Addition of lactose at varying concentrations to milk resulted in a reduction in pH and hence HCT; the extent of reduction in HCT was positively correlated with the level of lactose in milk (Crowley et al., 2014; Sweetsur & White, 1975). However, in the presence of lactose at low concentration, the stabilizing effect of lactose and urea on heat stability has been demonstrated (Kudo, 1980; Shalabi & Fox, 1982).

3.2.2.2. Effect of processing operations on heat coagulation time of milk

The effects of milk heat treatment on HCT of milk have been studied extensively (Huppertz, 2013; Mohammed & Fox, 1986; Singh & Creamer, 1991; Singh & Fox, 1985), though variations exit in the extent of changes in HCT after heat treatment of milk. Abundant studies have reported that heat treatment of milk did not affect the shape of HCT/pH profile (remained type A) but shifted the pH of HCT_{max} to a lower pH value, lowering the HCT_{min} and broadened the pH range of HCT_{min} (Sievanen, Huppertz, Kelly, & Fox, 2008; Singh & Creamer, 1991). The reduction in the pH of HCT_{max} in heated milk is considered to be associated with the

reductions in serum Ca, and hence [Ca^{2+}] and the increase in level of denatured whey protein with κ-casein linked *via* SH/S-S interchange. Increasing the pH prior to heat treatment of milk from 6.7 to 7.2 results in decreased pH of HCT_{max} from 6.5 to 6.4 (Singh & Fox, 1985).

HCT of milk with high total solids level has been measured at 120°C. Increasing TS of skim milk has a negative effect on HCT of skim milk. (Singh & Creamer, 1991) Singh and Creamer (1991) reported similar trend of progressive decrease in HCT as total solids increased from 9.7 to 20% in skim milk prepared by diluting concentrate or reconstituted skim milk powder. The impaired HCT on increasing total solids in milk is associated with the increasing volume fraction of casein, dissociation of κ-casein and level of lactose decomposing to organic acid (O'Connell & Fox, 2003). Heat treatment of milk before concentrating diminished the negative effect of increasing total solids on HCT. This effect of milk heat treatment is attributed to an increase in the proportion of denatured whey protein (86%) interacting with the casein micelle and a decrease in serum Ca (Huppertz, 2016; Singh & Creamer, 1991).

3.3. Ethanol stability (ES)

3.3.1. Determination and possible mechanism of ethanol-induced aggregation

The stability of milk to ethanol is a prerequisite in food applications containing both alcohol and milk, such as cream liqueurs, egg-nogs and other alcoholic beverages. (Abbott & Savage, 1985; Banks et al., 1982; Power, 1996).

The ES test of milk involves addition of ethanol to milk and observation of flocculation. A single-point ethanol stability test is conducted by blending equal volume of bovine milk at its natural pH with 70% (v/v) aqueous ethanol solution

(Chavez et al., 2004; Guo et al., 1998; Horne, 2016). The milk will be rejected if any clots or precipitates occur after addition of aqueous ethanol solution. This method has been employed in many countries, as an indicator of bovine milk or caprine milk for freshness (sourness), acceptance and processability, due to its simplicity, efficiency, speed and low cost (Horne, 2003).

Horne and Parker (1980) further investigated the ES of milk and reported a typical sigmoidal curve when ethanol stability was plotted against milk pH values ranging from 6.4 to 7.0, showing the increase in ES on increasing pH of milk. The ES test was modified by adding aqueous ethanol solutions with different concentration to milk. The ES of milk is defined as the minimum concentrations of aqueous ethanol solution (critical concentration) required to induce visible flocculation in milk. The method used for measuring ES was modified by increasing the volume ratio of ethanol to milk to 2:1 from 1:1 because of the failure to induce milk flocculation at high pH of milk (Horne & Parker, 1980). However, the variation in protein content in different milk samples is not considered in the modified method, leading to differences in the ratio of ethanol content to protein content. The constant volume ratio of ethanol to milk may result in inaccuracy and difficulties when comparing ES across different milk samples with varying protein content. A typical ES/pH profile is shown in Figure 1.2.

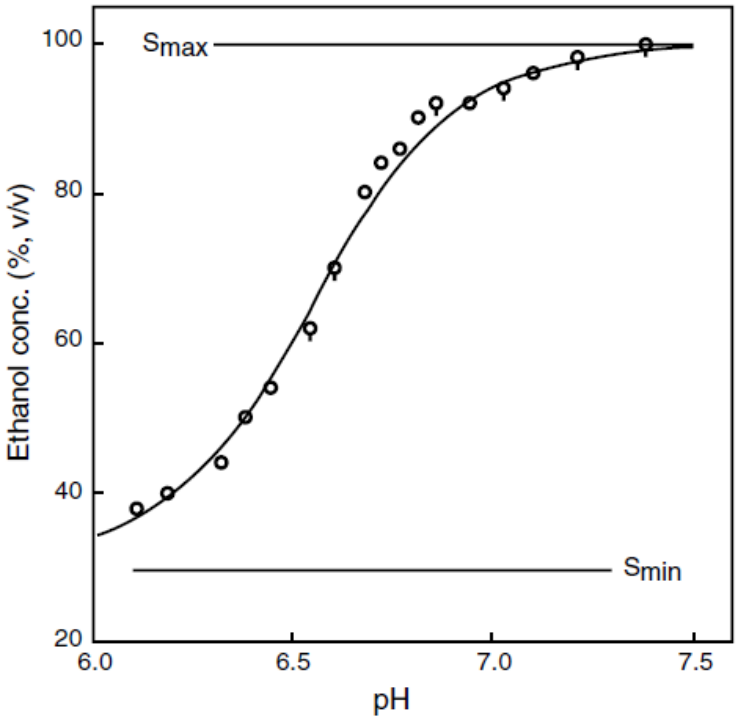

Figure 1.2. Effect of adjusting milk pH on the minimum concentration of ethanol solution required to coagulate the milk. Data adopted from Horne & Parker (1980).

A potential mechanism of ethanol-induced protein aggregation has been proposed by Horne (2003). Addition of ethanol to milk results in destabilization casein micelles by reducing the steric and electrostatic repulsion (Horne, 1984, 2003, 2016). Similar to the reduction in casein micelle size caused by hydrolysis of κ-casein following addition of rennet, Horne (1984) found a decrease in hydrodynamic radius of casein micelle at concentrations of added ethanol below the critical concentration of aqueous ethanol solution, followed by an increase in hydrodynamic radius as the concentration of ethanol exceeded the critical concentration. The changes in micelle size indicated the collapse of 'hairy layer' on the casein micelles, reduction in steric repulsion and subsequent aggregation of protein (Horne, 2016). The collapse of κ-casein is the result of reduced solvent quality on addition of ethanol (Horne, 2003). Rennet treatment of milk for varying length of time results in κ-casein with different levels of charge density. Titration of milk containing different

levels of charge density with ethanol until visible flocculation observed reveals the positive correlation between the ethanol stability and charge density of κ-casein. This confirmed the function of κ-casein as a salted polyelectrolyte brush (de Kruif, 1999). The ethanol-induced precipitation of calcium phosphate leads to reduction in free [Ca^{2+}], drawing away caseinate-bound calcium, increasing the thickness of the steric layer and casein micelle charge, all of which contribute to micelle stability. However, the response of stabilizing effect of precipitation of calcium phosphate to ethanol stability requires long time (Horne, 2003). When the rate of aggregation of protein occurs faster than the response for the adjustment of charge and conformation induced by precipitation of calcium phosphate, the aggregation process dominates and protein precipitates (Horne, 2016).

3.3.2. Factors affecting ethanol stability of milk

3.3.2.1. Physico-chemical and compositional factors affecting ethanol stability

As the proposed mechanism suggested, serum component and pH of milk play a significant role in ES.

Changing pH significantly affects the ES as shown in ES/pH profile (Figure 1.2). Increasing milk pH leads to reduction in solubility of calcium phosphate, with a stabilizing effect on the casein micelles (Horne & Parker, 1981a). Therefore, a higher concentration of aqueous ethanol solution is required to overcome the stabilizing effect and induce aggregation.

The soluble salt in the serum phase of milk is of particular importance in ES of bovine and caprine milk (Chavez et al., 2004; Guo et al., 1998; Horne & Parker, 1981a). The sigmoidal shape of ES/pH profile of milk was retained, despite varying levels and types of minerals present in the serum phase (Horne & Parker, 1981a).

The controlling role of the serum phase in ES has been demonstrated by the similar ES/pH profiles between original milk and re-formed casein dispersion in which casein from different milk was re-suspended in original milk serum (Horne & Parker, 1981a). Individual soluble minerals present in the serum phase of milk were investigated in previous studies, of which soluble Ca is the dominant factor controlling ES. Higher concentrations of ionic Ca or Mg reduced ES (Chavez et al., 2004; Horne & Muir, 1990; Horne & Parker, 1981a; Mohammed & Fox, 1986). Addition of equimolar phosphate anion and [Ca^{2+}] in milk restored ES in the pH range of 6.0 to 7.2, to an extent less than the ES in original milk. Soluble phosphate acts as a calcium-sequestrant, resulting in increased negative charge of casein micelle and an increased energy barrier to coagulation (Horne & Parker, 1981b). However, no significant effect on ES was observed on addition of 5 mM phosphate alone to milk whereas the positive effect of citrate on ES was dose-dependent, being noticeable when 5 mM citrate added (Horne & Parker, 1981a). Removal of calcium by high-pressure treatment of milk or addition of ethylenediaminetetracetic acid (EDTA) up to 5 mM to milk increased ES at pH 6.2 to 7.5 (Horne & Parker, 1981a; Huppertz et al., 2004; Mohammed & Fox, 1986). Alkali metal salts, e.g., NaCl, impaired the ES of milk at pH 6.6-7.5 (Horne & Parker, 1981a).

Chavez et al. (2004) reported higher levels of casein in milk samples stable to 72% (v/v) aqueous ethanol solution than these levels in unstable milk. The unexpected results may due to variations in protein to ethanol ratio in different milk samples when measuring ES using equal volumes of 72% ethanol solution and milk. Dissociation of κ-casein from casein micelle induced by high-pressure treatment of milk, leads to reduction in steric stability of casein micelles, which promoting the ethanol-induced aggregation in milk (Huppertz et al., 2004; Walstra, 1990).

Whey proteins have shown no effect on the ethanol-induced coagulation process, as shown by a lack of difference in casein dispersions prepared with or without whey proteins (Botaro et al., 2007; Horne & Parker, 1981a). Also, addition of lactose or urea at twice the natural concentration did not induce changes in ES (Horne & Parker, 1981a).

3.3.2.2. Processing factors affecting ethanol stability

As discussed earlier in this chapter, heat treatment of milk is a critical step used to increase the microbial safety, improve texture or properties in dairy products (e.g., yogurt), or improve the heat stability of concentrated milk. Previous studies have investigated the effect of heat treatment of milk on ethanol stability by heat treatment of skim milk or reconstituted skim milk at 90-120°C for 10 to 30 min (Horne & Parker, 1981c; Mohammed & Fox, 1986). The results showed an increase in ethanol stability in the pH range from 6.2 to 6.8 with increasing the severity of milk heat treatment, without significantly changing the sigmoidal shape of the ES/pH profile. Moreover, the ethanol stability at pH regions of 6.0 to 6.2 and 6.8 to 7.6 remained largely unchanged (Horne & Muir, 1990). The improvement in ES of milk at pH 6.2-6.8 was attributed to the heat-induced precipitation of calcium phosphate, which consequently reduced the concentration of serum Ca and [Ca^{2+}] in heated milk (Horne & Parker, 1981b; Mohammed & Fox, 1986). Heat-induced denaturation of whey proteins and their association with casein micelles when heated at native pH of milk did not affect ES (Horne & Parker, 1981c).

Relatively little information is available on effect of concentrating on ES of milk. Horne and Parker (1983) reported that the ES decreased at pH 6.7-7.5, as TS in skim milk increased from 9 to 23%, this effect being more pronounced as pH increased at ES test. They concluded that the negative effect of increasing total solids

on ES at pH >6.7 was due to the increase in chloride content, and hence ionic strength. It was hypothesised that higher ionic strength resulted in a shift in calcium-citrate equilibrium, which favoured a higher [Ca^{2+}] concentration, and hence lower ethanol stability, in high TS concentrates. However, at lower pH (<6.7), the ES of milk remained largely unchanged on increasing total solids in milk (Horne & Parker, 1983). It is important to note that the aqueous ethanol solution was added to milk at a constant volume ratio of 2:1 regardless of the differences in protein content in different samples. This may lead to inter-study discrepancy and inaccurate results in samples with different protein content. The combination of effects of heat treatment and concentration has not been investigated.

3.4. Acid-induced gelation of skim milk

Acidification of milk is the basis for dairy products (e.g., yogurt) and casein powder ingredients (e.g., lactic acid casein, mineral acid casein, sodium caseinate and calcium caseinate). In the manufacture of yogurt, acid in milk can be produced by fermentation of lactose to lactic acid by lactic acid bacteria (LAB). The optimum temperature for the growth of LAB, consisting of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, is 40-45°C (Tamime & Robinson, 2007). For manufacture of acid casein ingredients, milk is precipitated by lactic acid or mineral acid (e.g., hydrochloric or sulphuric acid). Milk can also be acidified by hydrolysis of added glucono-d-lactone (GDL) or injection of carbon dioxide (Lucey, 2016).

3.4.1. Changes in milk and formation of acid gel during acidification

The physico-chemical and rheological changes of milk during acidification are continuous and largely dependent on the pH of milk. When the pH of milk reduced from native pH (6.6-6.7) to 6.0, the electrostatic repulsion between caseins is decreased as a result of the reduction in negative net charge of casein micelles.

Limited amounts of CCP dissolve on decreasing milk pH to 6.0, but further reduction in pH from 6.0 to 5.0 leads to progressively decreases in electrostatic repulsion and steric stability of casein micelles. In contrast, reduction in pH results in maximum solubilisation of CCP at pH 4.9 (Dalgleish & Law, 1989). The solubilisation of CCP can weaken the internal structure of casein micelles and induce maximum dissociation of casein at pH 5.0-5.3 dependent on temperature (Dalgleish & Law, 1988). At pH decreases from 5.0 to 4.6, interactions between casein micelles increase due to the reduction in casein net charge, which decreases to zero at the pI of casein (pH 4.6). The hydrophobic interaction also contributes to the formation of acid gel containing clusters and chains of caseins (Lee & Lucey, 2010).

3.4.2. Factors affecting acid gelation and acid gel

In this section, the effects of heat treatment of milk and pH at heating prior to acid gelation, content of CCP in casein micelle and ionic strength on acid gelation, are discussed. Other important factors governing the formation of acid gel have been studied extensively, including pre-acidification of heated milk (Peng et al., 2009), rate of acidification (Lee & Lucey, 2004; Peng et al., 2009; Vasbinder et al., 2003), incubation temperature (Lee & Lucey, 2004; Lucey, van Vliet, Grolle, Geurts, & Walstra, 1997), casein concentration (Chever et al., 2014; Lucey, 2016), level of lactose (Meletharayil et al., 2016) and addition of stabilizers (Curtin et al., 1995; Guinee et al., 1995; Matia-Merino & Singh, 2007).

3.4.2.1. Heat treatment

During the manufacture of yogurt, milk is heated under temperature/time combination ranging from 85 to 140°C for 4 s to 30 min. The objectives of heat treatment of milk during yogurt manufacture include increasing microbial quality of yogurt, reducing undesired microorganisms and competition for the starter cultures,

removal of oxygen to assist the growth of starter cultures, and introducing a certain level of whey protein denaturation (Lee & Lucey, 2010; Lucey, 2016). Changes have been widely reported for the effect of milk heat treatment at native pH on the pH onset of gelation (pH_{og}) (Guyomarc'h, et al., 2009; Lucey et al., 1997; Vasbinder et al., 2003), gel firmness at pH 4.6 ($G'_{pH4.6}$) (Guyomarc'h et al., 2009; Lucey et al., 1997) and microstructure of the acid gels (Guyomarc'h et al., 2009; Lucey et al., 1998; Vasbinder et al., 2004).

Heat treatment of milk resulted in association of denatured whey protein (mainly β-Lg) and casein micelle (Vasbinder et al., 2003; Vasbinder & de Kruif, 2003). The increases in pH at gelation onset (GOT_{pH}) and $G'_{pH4.6}$ may be attributed to the higher isoelectric point (pI) of β-Lg (~5.3) and 'bridging' role of denatured whey proteins for interactions of casein particles, respectively. The involvement of denatured whey protein in acid gel, which has been confirmed by confocal laser scanning microscopy (Vasbinder et al., 2004), increases the number and strength of bonds between protein particles to form an acid gel with enhanced inter-connectivity in heated milk (Guyomarc'h et al., 2009; Lucey et al., 1998). Maximum loss tangent (d_{max}) is observed in heat-treated milk with higher GOT_{pH} (>pH 5.2) in the range of pH at which CCP solubilisation occurs. Loss tangent increases when the gel network is partially loosened as solubilisation of CCP increases. The decrease in d as pH decreases has been attributed to the decrease in electrostatic repulsion between caseins by reducing net charge, and increases in protein interactions. (Lucey, 2016; Lucey et al., 1998). Simultaneously, a change in slope of G' ($G'_{shoulder}$) at pH similar to the pH at which d_{max} occurs, especially at incubation temperature >40°C (Anema, 2009). This may be associated with the dissolution of CCP followed by increased

protein-protein interactions as pH decreases (Anema, 2009; Lee & Lucey, 2004; Meletharayil et al., 2015).

3.4.2.2. Effect of pH at heat treatment on acid gelation

The effects of pH at heating on acid gelation has been reviewed and studied widely (Anema, Lowe, & Lee, 2004; Donato, Alexander, & Dalgleish, 2007; Donato & Guyomarc'h, 2009; Guyomarc'h, Jemin, Le Tilly, Madec, & Famelart, 2009; Lakemond & van Vliet, 2008a, 2008b). At pH < 6.7, heat-induced denatured whey protein attaches to the casein micelles. As the pH at heating is increased (e.g., 6.5 to 7.1), κ-casein dissociates increasingly from the micelle and complexes with denatured whey proteins in the serum to form serum-phase aggregates (Anema, 2007; Anema & Klostermeyer, 1997). It has been observed that pH_{og} of milk increased with pH (from 6.35 to 7.1) prior to heat treatment (Anema et al., 2004; Lakemond & van Vliet, 2008a; Vasbinder & de Kruif, 2003). Vasbinder and de Kruif (2003) indicated that the presence of whey protein aggregates in serum at higher heating pH (>6.7) influenced the GOT_{pH}. Additionally, Lakemond and van Vliet (2008a,b) suggested a reduction in steric hindrance due to the lower level of denatured whey protein associated with casein micelle when milk was heated at higher (pH >6.7), which may favour the interaction between casein micelles, and thus increased pH_{og} in heated milk. As a result of κ-casein dissociation on heating of milk at higher pH values (pH >6.7), the κ-casein-depleted casein micelles may collapse or become less stable due to the reduction of density of the surface hairy layer governing the stability of casein micelle (Anema et al., 2004).

4. Effect of seasonality on milk composition and functional properties

There are two types of commonly used cow herd management systems: year-round calving herd and compact calving herd. The cows from the former system calve regularly throughout the year, and therefore enable even milk production during a year. The cows in the latter herd calve at a specific time (e.g., February to March in Ireland) of year and the production of milk is from February to November or December, with the increasing in milk production in April and May and decreasing afterwards until the end of lactation in November or December (O'Brien & Guinee, 2011; O'Brien et al., 1999a). The impact of the herd management systems is reflected by the changes in composition and processability of milk influenced by the stage of lactation. Milk supply from compact calving herd containing spring- or autumn-calved milk as predominate portion, is expected to have larger variation in milk composition (Davies & White, 1958; White & Davies, 1958), which may be undesirable due to the resultant variation in functional properties (Auldist et al., 1996; Cheng et al., 2002; Donnelly & Horne, 1986; Guinee et al., 2007; Horne et al., 1986; Kelly et al., 1982; O'Keeffe et al., 1982). Seasonal variations in compositional, physico-chemical and functional properties of milk has been a topic of interest and world-widely reported (Auldist et al., 1996; Barry & Donnelly, 1980; Chen et al., 2014; Cheng et al., 2002; Donnelly & Barry, 1983; Donnelly & Horne, 1986; Glantz et al., 2010; Holt & Baird, 1978; Holt & Muir, 1978; Holt et al., 1978; Horne et al., 1986; Kelly et al., 1982; Keogh et al., 1982; Lindmark-Månsson et al., 2003; O'Brien et al., 1999a,b; O'Keeffe et al., 1982; Phelan et al., 1982).

4.1. Seasonal changes in composition and physico-chemical properties of milk

The protein and casein content, which are the major components providing nutritional and functional values in milk, vary from 2.89 to 3.72% (w/w) and 2.08 to 2.92%, respectively, in bulk milk from year-round calving herd and retail milk (Chen et al., 2014; O'Brien et al., 1999a; Phelan et al., 1982). For the milk from spring-calved herd or manufacturing milk mostly consisting of spring-calved herd, the protein and casein content ranged from 2.87 to 4.27% (w/w) and 2.19 to 2.88 %, respectively (Mehra et al., 1999; Phelan et al., 1982). The levels of protein and casein increase towards the end of milk lactation (Gulati et al., 2017). Individual caseins in milk are also affected by the lactation stage of cow. In milk obtained from winter/spring-calved herd, the levels of α_s- and β-casein were found decreased from middle lactation to late lactation by 13 and 10% of casein in milk. respectively (Barry & Donnelly, 1980). The study by Donnelly and Barry (1983) reported the gradual reductions in α_s- and β-casein from 54 and 40 in early lactation, to 45 and 27 (% casein in milk) towards the end of lactation, respectively, in manufacturing milk. Additionally, the proportion of κ-casein (% casein in milk) remained constant at a level of 9-10% throughout the year. Serum casein, as a % of milk casein, was 8% for fresh milk at 20°C (Dalgleish & Law, 1988). The effect of season on serum nitrogen fraction is relatively less studied.

Mineral concentration in milk changes with season and milk lactation. Levels of Ca and P ranged from 98-126 and 80-102 in milk from mixed herd, liquid milk or bulk milk from dairy plants (Chen et al., 2014; Keogh et al., 1982; Lindmark-Månsson et al., 2003). In milk from spring-calved cows or manufacturing milk containing milk mainly from spring-calved cows, Ca and P content varied widely from 108 to 151,and 55 to 112 mg/ 100 g (Gulati et al., 2017; Keogh et al., 1982;

O'Brien et al., 1999b; Sola-Larrañaga & Navarro-Blasco, 2009; White & Davies, 1958). In the recent study of Gulati et al. (2017), total Ca and P contents was observed to be significantly affected by lactation period, which was higher in late lactation than that in the middle lactation in milk from spring-calving. Serum Ca and P accounted for 26 to 33%, and 25 to 53% of total in spring-calving milk. Serum Ca decreased significantly whereas no definite trend is found in serum P (Gulati et al., 2017; Keogh et al., 1982). The inter-study discrepancy may be attributed to the different method, temperature and pH of determination, and the genetic difference between cows. The changes in level of other trace elements in milk were studied by (Gulati, et al., 2017; Keogh et al., 1982; O'Brien et al., 1999b; Sola-Larrañaga & Navarro-Blasco, 2009).

The pH of milk is significantly affected by season, as the pH varied from 6.41 to 6.85 in bulk milk from year-round calving herd, retail milk (Chen et al., 2014; Phelan et al., 1982). Similarly, in the spring-or autumn-calved milk, pH varied from 6.53 to 6.83 throughout the year (Guinee et al., 2007; Phelan et al., 1982). Casein micelle size in bulk-milk from creamery was affected by season, which is significantly higher in June to August than in December to February. An opposite trend was found by (Glantz et al., 2010) who reported significant increase in casein micelle in December to February. In the milk from spring-calved Ayrshire cows, casein micelle size decreases as lactation stage progresses (Holt & Baird, 1978).

4.2. Seasonal changes in functional properties of milk

In addition to compositional and physico-chemical characteristics, functional properties of milk, such as rennet gelation properties, heat stability and ethanol stability, are also affected.

Rennet gelation time in bulk milk from year-round calving herds, measured at native pH of milk, was significantly higher in March to May (~21 min) than that in September to November (~17 min) (Chen et al., 2014), which would imply considerable differences in rennet-curd cheese manufacture. The difference in rennet gelation time may be associated with pH of milk, which is significantly higher in Spring (March to May) than that in the other time of the year (Chen et al., 2014). However, no significant different was observed in rennet gelation time and gel firmness in liquid milk renneted at pH 6.6 (O'Keeffe et al., 1982). Heat stability of bulk liquid milk was determined by its heat coagulation time at native pH of milk at 130°C. The heat stability of liquid milk collected in December to March was the lowest throughout the year (Kelly et al., 1982). In the manufacturing milk, the high heat stability of milk collected from June to October may associated with the higher urea content in milk (Holt et al., 1978). The trend of seasonal effect on ES at native pH of milk is similar to the trend in rennet gelation time, with higher ES of milk in March to May than that in September to November (Chen et al., 2014). Effect of lactation stage on ES has been observed in spring-herd milk (Davies & White, 1958; Horne et al., 1986). In the early stage of lactation, milk is very unstable to ethanol due to the high level of Ca^{2+} present in milk (Tsioulpas et al., 2007). The ES/pH profile shifted to alkaline side as lactation progressed. Moreover, at pH 6.6, the ES decreased through lactation (Horne et al., 1986). This might be attributed to the lower in serum Ca in middle lactation than that in late lactation (Gulati et al., 2017).

5. Dairy powder ingredients

Milk-derived protein powder ingredients have been customized to meet the specific requirements on nutritional and functional properties, for instance in bakery products, formulated food, ice-cream, beverages, energy drinks, coffee whitener and

other applications. The typical composition of selected milk protein powder ingredients is shown in Table 1.4.

Table 1.4. Gross composition of different types of some dairy protein powder ingredients. Data obtained from Deeth and Hartanto (2009) and Guinee et al. (2009).

	Skim milk powder	Sodium caseinate	Calcium caseinate	Native phosphocasein
Moisture (%, w/w)	3-5	3-5	3-5	3-5
Protein (%, w/w)	35-37	89-95	89-95	74-80
Lactose (%, w/w)	49-52	0.2	0.2	0.2
Ash (%, w/w)	7.5-8.0	3.5-5	3.5-5	3.5-5
Calcium (mg/100g)	1183-1260	100	1000-1500	2000-2400
Phosphorus (mg/100g)	970-1103	800	800	1000-2500
Sodium (mg/100g)	428-530	1200-1400	50-100	50-100
Fat (%, w/w)	0.7-1.3	0.9-1.5	0.9-1.5	<2

5.1. Skim milk powder (SMP)

SMP is classified as low-, medium- or high-heat SMP according to the heat treatment applied to skim milk prior to evaporation and drying (Martin et al., 2007). Typical heat treatments are 70-72°C for 15 s for low-heat SMP, 85°C for 120 s for medium-heat SMP, and 120°C for 60-120 s, or 90°C for 300 s for high-heat SMP (Kelly et al.,2003). For all types of SMP, the stages of manufacture include heat treatment of the milk, evaporation to ~45-50% total solids, and spray drying to ~97% TS. Heat treatment, depending on the severity (temperature and time) and milk pH, affects the extent of whey protein denaturation, the binding of denatured whey protein to the casein micelle, and the partitioning of components (salts, whey protein and caseins) between the serum and micellar phases of milk (Donato & Guyomarc'h, 2009).

Low-heat powder is also used extensively in food formulation, including applications such as recombined milk for cheese manufacture, milk solids standardization in products such as cheese milk, yoghurt and fermented milk

products (Patel et al., 2007). High-heat SMP is used as an ingredient in bakery, sweetened condensed milk, and confectionery products such as UHT recombined concentrated milk, toffee, caramel, fudge and milk chocolate (Aitken et al., 1999; Stewart et al., 2017).

5.2. Native phosphocasein (NPC)

Developments in membrane filtration of milk since the 1970s (Maubois et al., 1969) have led to development of an array of high protein powders. NPC can be prepared by microfiltration (MF) of skim milk, diafiltration (DF) to ~16% TS, and spray drying to ~97% TS. Calcium-reduced phosphocasein can be manufactured from MF skim milk containing calcium-sequestrants (e.g., a mixture of tri-, di- and mono-sodium citrate with citric acid; potassium salts of citric acid; sodium and potassium salts of food grade organic acids including lactic, acetic and oxalic acids; and sodium or potassium salts of phosphates, tri-sodium citrate and citric acid), DF to ~16% total solids, and spray-drying to ~97% total solids (Guinee et al., 2009; Kelly et al., 2000). Micellar caseins comprise high protein level (mainly casein), minerals, very little whey protein and lactose. Native phosphocasein has good rennet coagulability (Rollema & Muir, 2009). The applications of this powder include standardization of cheese milk (Guinee et al., 2006) and formulation of processed cheese (Guinee, 2009).

5.3. Sodium caseinate and calcium caseinate (CaCas)

Acid casein is manufactured by acidification of skim milk to pH 4.6, followed by washing and drying. The solubility of acid casein is poor in water, and as a result, its applications in food are limited. In contrast, NaCas and CaCas, obtained by addition of NaOH or $Ca(OH)_2$ to acid casein and spray-drying, are highly soluble in water. Caseinates are non-micellar high protein ingredients

containing very little whey protein. Sodium caseinate is very stable to ethanol and heat treatment at 140°C for 1 h. Calcium caseinate has poor heat stability. (O'Kennedy et al., 2001; O'Kennedy et al., 2006). NaCas is found in many applications including processed cheese spreads, cream liqueurs, coffee whiteners and bakery products (Abbott & Savage, 1985; Guinee, 2009; Lynch & Mulvihill, 1997). Calcium caseinate is suitable for formulation of nutritional beverages (Lagrange et al., 2015).

5.4. Milk protein concentrates (MPC)

5.4.1. Manufacture and applications

An attempt to produce MPC was first made around 1990; which has been classified as a second generation dairy ingredients (Meena et al., 2017).

MPC powders are commercially available from 45 to 85% protein on the dry weight basis. The protein content is denoted as part of their names (e.g., MPC80 contains 80% protein). Protein in MPC powder is purified by removal of NPN, lactose and soluble salts, using ultrafiltration alone or in combination with diafiltration, followed by evaporation and spraying-drying or spray-drying only. MPC45-65 can be manufactured by ultrafiltration of pasteurized skim milk, evaporation of retentate and spray-drying, while the production of MPC70-85 requires the diafiltration of retentate obtained on ultrafiltration, followed by spray-drying (Crowley et al., 2014). The current chapter focuses on the high protein MPC containing protein ≥80%.

MPCs are used extensively in food manufacture/formulation, with applications including dairy-based beverages, yoghurt, fresh-cheese products, recombined milk cheeses, ice-cream, coffee whitener, and high protein bars

(Agarwal et al., 2015; Alvarez et al., 2005; Ikeda, 2015; Loveday et al., 2009; Meena et al., 2017; Patel et al., 2014; Uluko et al., 2016). In these applications, MPC is required to provide the requisite functionalities or combinations thereof, including emulsification, rennet gelation properties, acid gelation properties, foaming, heat stability and/or nutritive value. High protein MPC powders are more functional that other ingredients, such as SMP or whey protein concentrates, in many applications owing to the combined functionalities of both casein and whey protein, their neutral flavour (for example compared to sodium caseinate), and low lactose content (<3%). Lactose is a non-functional ingredient in many formulations and high levels increase formulation cost, the risk of crystal formation in products such as ice cream, and browning in products subjected to high temperature conditions during manufacture (e.g., UHT products) or food service (e.g., formulated foods that are baked/grilled). In respect of nutritional value, high lactose content is also undesirable.

5.4.2. Functional properties of milk protein concentrates

A number of recent studies have reported the functionality of high protein MPC affected by manufacturing conditions or process including:

- heat treatment of the skim milk prior to UF (Crowley et al., 2015; Gazi & Huppertz, 2015)
- high-pressure treatment of the skim milk prior to UF (Udabage et al., 2012)
- homogenization, microfluidization, ultrasonication of retentate before spray-drying (Augustin et al., 2012)
- the use of extrusion personification process, instead of spray-drying (Bouvier et al., 2013)
- alteration of Ca content by

- pre-acidification (Eshpari et al., 2017)
- reduction in temperature from 40 to 20°C of the skim milk prior to UF and/or DF (Liu et al., 2014)
- addition of NaCl (Mao et al., 2012)
- addition of calcium-chelating salts to the retentate (Bhaskar et al., 2001)

Rennet coagulability, heat stability and acid gelation properties of MPC is discussed in this review. Other functional properties, with equal importance in applications of MPC, that have been investigated or reviewed in the literature include solubility, emulsification and fomability (Bouvier et al., 2013; Euston & Hirst, 2000; Huppertz & Gazi, 2015; Luo et al., 2015; Luo et al., 2016; Mao et al., 2012; Ye, 2011). However, studies on stability of MPC to ethanol are scarce, though it might be considered as a critical property and could be potentially used in alcohol-based beverages and foods.

5.4.2.1. Rennet-induced gelation

As discussed in Section 3.1.1, rennet-induced gelation is the fundamental step in manufacture of rennet-curd types of cheese, such as Cheddar and Mozzerlla. MPC powder is increasingly used for protein extension during manufacture of cheese to improve rennet coagulability and final cheese yield (Casiraghi et al., 1989; Shakeel-Ur-Rehman et al., 2003). A firmer rennet gel was obtained in skim milk fortified with MPC (non-dialyzed fortified skim milk) compared to those fortified with MPC and then dialyzed against skim milk; the firmer gel resulted from a higher insoluble Ca content in the non-dialyzed fortified skim milk (Ferrer et al., 2008).

Increasing heat treatment of milk prior to UF/DF or recombined milk significantly impairs rennet coagulability of reconstituted or recombined milk. (Carr, 1999) prepared MPC85 powders with different levels of whey protein denaturation

by heat treatment of skim milk. A reduction in gel strength was observed in MPC dispersions as heat treatment of skim milk increased, especially in dispersion prepared from MPC85 with ≥48% whey protein denaturation. (Pomprasirt et al., 1998) investigated the effect of heat treatment on the course of gel formation in recombined milk containing 20% anhydrous milk fat and 20% MPC powder. Increasing heat treatment on recombined milk resulted in reductions in gel firming rate, gel firmness at 2 h after addition of rennet. The degree of increase in rennet gelation time is dependent on the severity of heat treatment.

Rennet gelation properties was reported to be poor in protein dispersions reconstituted from commercial MPC powders (Kuo & Harper, 2003) or low-heat MPC powder manufactured at pilot plant at different research centres (Ferrer et al., 2008; Martin et al., 2010). The poor performance in rennet gelation of MPC dispersion is largely attributed to the insufficient serum calcium content or the low calcium to casein ratio. The restoration of rennet coagulability can be achieved by supplementing with at least 2 mM $CaCl_2$ or dialysis against skim milk to obtain the composition of serum phase in skim milk (Ferrer et al., 2008; Martin et al., 2010).

5.4.2.2. Heat stability

Increasing severity of milk heat treatment from 72°C for 15 s to 95°C for 45 s led to an increase in the level of denaturation of total β-Lg to 65% and total α-Lac to 25% (Gazi & Huppertz, 2015), but had little impact on the heat coagulation time of aqueous dispersions of the MPC (8.5% protein) at 120°C in the pH range 6.3-7.1 (Crowley et al., 2015). In MPC dispersions with 3.5% protein, MPC dispersions reconstituted in water exhibited a HCT/pH profile similar to that of type B milk, for which HCT increase as pH increases from ~6.6 to 7.4. However, HCT in type B milk

increases as pH increases from 6.2 to 7.2, whereas the aqueous-based MPC dispersion is very susceptible to heat at pH from 6.2-6.6, evidenced by HCT <2 min (Carr, 1999; Crowley et al., 2014). Increasing severity of milk heat treatment prior to concentration, with resulting increases in extent of when protein denaturation from 0 to 92.3% in MPC powder, resulted in significant decrease in HCT at pH region of 6.6 to 7.2 when the whey protein denaturation level was ≥91% (Carr, 1999). Compared with rennet gelation, heat stability of MPC dispersion is less sensitive to whey protein denaturation of MPC powder.

Reconstitution of low-heat treated MPC80 using simulated milk ultrafiltrate (SMUF) or SMUF with lactose (4.6%) and urea (30 mg/ 100 g) resulted in induction of maximum HCT at pH 6.7-6.8. Type A HCT/pH profile of low-heat MPC dispersion was restored by cold dialysis of aqueous MPC dispersion against skim milk (Figure 1.3) (Crowley et al., 2014).

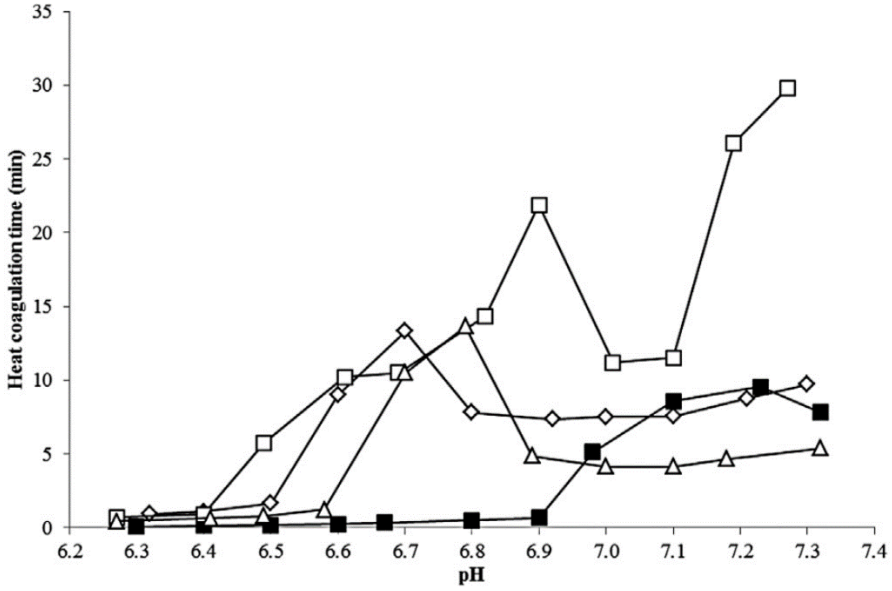

Figure 1.3. HCT/pH profile at 140°C for milk protein concentrate with 80% protein reconstituted in water (■), simulated milk ultrafiltration (SMUF;◇), SMUF fortified with 4.62% (w/w) lactose and 300 mg/L urea (△), or milk protein concentrate dispersion dialysed against reconstituted skim milk (□). Data adapted from Crowley et al. (2014).

5.4.2.3. Acid-induced gelation and ethanol stability

In manufacture of yogurt, MPC can be added to skim milk or reconstituted skim milk as stabilizer in the formulation (Guinee et al., 1995; Karam et al., 2013). The acid gelation properties of MPC dispersion has been less investigated.

Meletharayil et al. (2016) studied acid gelation induced by hydrolysis of GDL in MPC dispersion (4% protein) prepared in water increasing lactose content from 0 to 5.6% or 11.2%. Higher lactose content in low-heat MPC dispersion led to an increase in GOT_{pH} and $G'_{pH4.6}$ of MPC dispersion but a reduction in the water-holding capacity of acid gel in the presence of lactose, which may associated with higher levels of non-sedimentable κ-casein and whey proteins. Previous results suggested the marked effect of solvent composition on heat-induced dissociation of κ-casein and on casein-whey protein interactions.

Previous studies have highlighted the important roles of heat treatment and solvent composition in rennet coagulability and heat stability of MPC dispersions; nevertheless, no information is available on the combined effects of heat treatment and solvent composition on rennet gelation, HCT and acid gelation of MPC dispersions.

Conclusion

This chapter reviews the current understanding of milk composition and the mechanism of functional properties of milk, including rennet-induced gelation, heat stability, ethanol stability and acid-induced gelation. A fundamental knowledge of how the milk compositional and physico-chemical proprieties (protein, minerals, lactose and pH) and processing operations (heat treatment and concentration) of milk affecting various functionalities is necessary for food formulation. High-protein

dairy ingredients are widely used as nutritional and functional ingredients in the food industry. Such knowledge of functional properties obtained from milk could give indications in the design and optimization of the manufacture and reconstitution conditions of milk-derived protein powder ingredients with desired functional properties for applications such as nutritional beverages, cream liqueurs, food formulations, and recombined milk for manufacture of cheese and fermented milk products.

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Objective

The overall objective of this work was to investigate the changes in compositional, physico-chemical and functional characteristics of dispersions of dairy powders, and how these are affected by seasonality, heat treatment, evaporation and spray-drying during powder manufacture, pH adjustment, modifications to solvent composition, type of added protein ingredients, and level of the added protein ingredients on protein fortification. The specific sub-objectives of the work presented in this thesis were:

- To understand the effect of seasonality on mixed herd skim milk from spring-calved and autumn-calved cows on composition (i.e., total solids, lactose, total protein, casein, individual casein, whey protein, non-protein nitrogen, calcium, phosphorus, serum protein, serum casein, individual casein dissociation, serum calcium, and serum phosphorus), physico-chemical (i.e., pH, casein hydration, casein micelle size, protein charge) and functional properties (i.e., rennet gelation, heat stability and ethanol stability) of milk and to establish the possible relationship between the former and the latter (Chapter 2);
- To characterise the composition of milk protein ingredients (i.e., skim milk powder, sodium caseinate, calcium caseinate, native phosphocasein and calcium-reduced phosphocasein) and to study the effects of different types of milk protein ingredients used for fortification of protein content on the compositional, physico-chemical and functional properties of skim milk (Chapter 3);
- To investigate the effect of different levels of addition of sodium caseinate to skim milk for protein fortification on the compositional and physico-chemical

characteristics, rennet-induced proteolysis, and functional properties, of skim milk (Chapter 4);

- To evaluate the effects of heat treatment of skim milk, evaporation and spray-drying during manufacture of skim milk powder on the compositional, physico-chemical and functional properties of raw skim milk, re-diluted skim milk from skim milk concentrates, reconstituted skim milk and skim milk concentrates prepared using skim milk powder (Chapter 5);
- To determine the effects of high-heat treatment of skim milk, and pH at heating before restoration of pH to 6.6, on the compositional, physico-chemical and functional properties of skim milk (Chapter 6);
- To examine the effects of heat treatment of skim milk during subsequent manufacture of milk protein concentrates (MPC) and the solvent composition used for reconstitution of MPC on composition, physico-chemical and functional characteristics (i.e., rennet gelation, heat stability, ethanol stability and acid gelation) of MPC protein dispersions (Chapter 7).

Chapter 2

Seasonal variation in the composition and processing characteristics of herd milk with varying proportions of milk from spring-calving and autumn-calving cows

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Abstract

This study investigated the seasonal changes in the compositional, physicochemical and processing characteristics of milk from a mixed herd of spring- and autumn-calving cows during the year 2014-2015. The volume proportion of autumn-calving milk (% of total milk) varied with season, from ~ 10-20 in Spring (March–May), 5-13 in Summer (June-August), 20-40 in Autumn (September-November) and 50-100 in Winter (December-February). While all characteristics varied somewhat from month to month, variation was inconsistent, showing no significant trend with progression of time (year). Consequently, season did not significantly affect many parameters including concentrations of total protein, casein, whey protein, NPN, total calcium, pH, rennet gelation properties or heat stability characteristics. However, season had a significant effect on the concentrations of total P and serum P, levels of α_{s1}- and β-caseins as proportions of total casein, casein micelle size, zeta potential and ethanol stability. The absence of a significant effect of season for most compositional parameters, rennet gelation and heat-stability characteristics suggest that milk from a mixed-herd of spring- and autumn-calving cows is suitable for the manufacture of cheese and milk powder on a year-round basis, when the volume proportion of autumn milk, as a % of total, is similar to that of the current study.

Key words: Milk, seasonality, composition, heat stability, ethanol stability

2.1. Introduction

Seasonal variation in the composition of bovine milk has been widely reported (Auldist et al., 1998; Chen et al., 2014; O'Brien, et al., 1999a,b). Contributing factors include stage of lactation, nutrition, health status, lactation number, and proportions of cows in a herd calving at different times of the year (Guinee & O'Brien, 2010; O'Brien & Guinee, 2011). The most extensively used feeding methods for dairy cows include indoors offered total mixed ration, comprised mainly of silage, grain, protein and added vitamins and minerals, or outdoors grazing on pasture, usually with a low quantity of concentrate supplementation offered only at the extremes of the pasture-growing season. The former is used most extensively, for example in continental Europe, USA, China, North Africa and India, while outdoors grazing on pasture is preferred in regions where the climate is more temperate and grass growth is abundant, especially in Ireland, New Zealand and South Eastern Australia. With outdoor grazing, dairy herds may consist of cows that calve over a relative short period in spring (compact calving), or alternatively of cows that calve more regularly throughout the year (year-round calving). These herd types coincide with two milk production systems from pasture, the latter with more consistent milk production throughout the year, and the former with a peak milk production at a specific time of year (e.g., April–May) and decreasing steadily until end of lactation (e.g., December). A major difference between these pasture-feeding systems is that stage of lactation can have a significant impact on the composition and quality characteristics of a milk supply obtained solely, or largely, from spring-calved herds but not on the characteristics of a milk supply from year-

round calving-herds (Davies & White, 1958; White & Davies, 1958a). Despite this limitation, milk from pasture-fed, spring-calving herds predominates in some countries, such as Ireland and New Zealand, as it provides the most cost-effective feeding system.

The relatively large seasonal variation in the composition of milk from spring-calving herds is undesirable as it can lead to variation in the stability characteristics of milk, e.g., heat stability (Kelly et al., 1982; White & Davies, 1958b), ethanol stability (Davies & White, 1958), rennet gelation characteristics (O'Brien et al., 1999a), and in the composition, quality and yield of dairy products such as cheese (Auldist et al., 1996; Guinee et al., 2007; Kefford et al., 1995) and yoghurt (Cheng et al., 2002).

To minimize the variation in composition and quality of milk associated with stage of lactation in pasture-based feeding systems without a consistent year-round calving pattern, processors sometimes use a blend of milk from spring- and autumn-calving herds, whereby the use of milk from the extremes of lactation in each supply (spring- or autumn-calving) can be avoided, while allowing continuity of supply across the year. However, relatively little has been published on seasonal variation in the composition or processability characteristics of a milk supply based on spring- and autumn-calving herds. Hence, the objective of the study was to monitor the seasonal changes in the compositional, physicochemical, rennet gelation, heat stability and ethanol stability characteristics of milk from a pasture-based mixed herd in which the proportions of milk from spring-and autumn-calving cows varied.

2.2. Materials and methods

2.2.1. Chemicals

The following chemicals were used: lanthanum chloride, hydrochloric and glacial acetic acid, trichloroacetic acid (TCA), acetic acid, sodium acetate (Thermo Fisher Scientific, Pittsburgh, PA, USA); calcium (Ca, 1,000 ppm) and phosphorus (P, 1,000 ppm) standard solutions for atomic absorption spectroscopy, L-ascorbic acid, sodium molybdate (≥98%), urea, acetonitrile, trifluoroacetic acid, α_S-casein (α_{S1} and α_{S2}-casein were quantified together), β-casein, κ-casein, and sodium azide (Sigma-Aldrich, St. Louis, MO, USA); 2-mercaptoethanol and sulphuric acid (VWR International, Dublin, Ireland). Ethanol (99.9%) was obtained from Carbon Group (Cork, Ireland).

2.2.2. Milk preparation

A representative milk sample (~2 L) was collected monthly from the mixed spring-calving/autumn-calving Holstein Friesian herd at the Teagasc Animal and Grassland Research and Innovation Centre Moorepark, over the period March 2014 to November 2014 and January 2015 to February 2015. The mean calving date for the spring-calving herd was 10 and 11 February in 2014 and 2015, respectively; that for the autumn-calving herd was 5 September in both 2013 and 2014. All milk samples, which comprised a bulked mixture of evening and morning milk, were cooled en route to the bulk tank, maintained at 4°C, sampled within ~3 h of the morning milking, and analysed within 48 h.

The proportions of milk changed over the year (Figure 2.1a; Table 2.1), with milk from the spring-calving herd decreasing from a maximum of ~95% in August to ~0% in January. Simultaneously, milk from the autumn-calving decreased from

~100% of total milk in January to ~5% in August. The variation in proportions of milk from spring- and autumn-calved cows coincided with changes in the number of autumn- and spring-calving cows over the year, as shown in Figure 2.1b.

2.2.3. Composition of raw milk

Milk was analysed in duplicate for total solids, total protein, fat and lactose using the FOSS MilkoScan™ FT+ analyser (Foss Electric A/S, Hillerød, Denmark). The composition of the raw milk is shown in Table 2.1.

2.2.4 Preparation of skim milk

Raw milk was heated to 40°C for 30 min, skimmed to ~ <0.1% (w/w) fat using a disc bowl centrifuge (FT15 Disc Bowl Centrifuge, Armfield Limited, Ringwood, UK), preserved using sodium azide (0.2%, w/w), and held at 4°C until required for analysis. Skim milk was used in preference to whole milk, as the presence of fat can complicate many of the analyses undertaken such as protein profiling, casein micelle size and hydration and heat stability; consequently, milk is typically defatted prior to these analyses (Dalgleish & Law, 1988; Glantz et al., 2010; Holt et al., 1978; Huppertz, 2016).

2.2.5. Preparation of skim milk serum

Milk serum was prepared by ultracentrifugation of skim milk at 100,000 *g* at 25°C for 1 h (Sorvall Discovery 90SE ultracentrifuge, Kendro Laboratory Products, Asheville, North Carolina, USA) using a fixed–angle Sorvall Titanium-1270 rotor (Dalgleish & Law, 1988) and filtration of the centrifugate through glass-wool.

2.2.6. Gross composition and physicochemical analysis of skim milk and skim milk serum

Skim milk was analysed for lactose using a FOSS MilkoScan™ FT+ (Foss Electric A/S, Hillerød, Denmark) and fat and total solids using the CEM SMART Trac II (CEM, North Carolina, USA) and pH.

Ca and P were determined on the ash prepared by drying the milk or serum sample (3 g) on a hot plate (CERAN 500, NiCr-Ni Electronic, Angermund, Germany) for 1 h followed by ashing at 550°C for 16 h. The ash was diluted with 3 mL HCl (3 M) and made to 100 mL in distilled water. A sample (1 mL) of the dissolved ash was mixed with 1 mL lanthanum chloride (10%, w/v) and made to 100 mL with distilled water. The Ca content was determined using atomic absorption spectrometry (AA240, Varian AA, Varian Inc., CA, USA) at 422.7 nm (IDF, 2007). A standard curve of absorbance at 422.7 nm versus Ca content was prepared by appropriate dilution of the calcium standard solution (1000 ppm) to give solutions ranging in Ca content from 0 to 0.25 mg/100 mL.

A sample (1 mL) of the dissolved ash was mixed with 20 mL molybdate/ascorbic acid solution and made to 50 mL with distilled water. The phosphorous was measured using molecular absorption spectrometry (Genesys^{TM5}, Milton Roy, PA, USA) at 820 nm (IDF, 2006). A standard curve of absorbance at 820 nm versus phosphorus content was prepared by dilution of the phosphorous standard (1000 ppm) to give solutions ranging from 0 to 0.2 mg/100 mL.

Total nitrogen (N) content was determined using the Kjeldahl method (IDF, 2001a). Non-casein nitrogen (NCN) was measured on a filtrate prepared by adjustment of milk or serum to pH 4.6 using sodium acetate/acetic acid buffer, according to the IDF standard method (IDF, 2004). The non-protein nitrogen (NPN) content was measured on a filtrate prepared by mixing milk or serum and 15% trichloroacetic acid at a volume ratio of 1:4 (IDF, 2001b). Casein N as a percentage of total N was calculated using the following formula:

Casein N = 100 - NCN, where, NCN is expressed as % of total N.

Whey protein N, as a % of total N, was calculated using the equation:

WPN = 100 - Casein N - NPN, where, NPN is expressed as % of total N.

The individual proteins were analysed by reversed-phase high pressure liquid chromatography (RP-HPLC), according to the method of Visser et al. (1991) with slight modifications. Protein calibration standards for RP-HPLC included α_s-casein, β-casein, κ-casein, α-lactalbumin (α-Lac), β-lactoglobulin (β-Lg) and whey protein isolate. Milk or milk serum was diluted 1:20 and 1:5 (v/v), respectively, with fresh pH 7.5 buffer containing 7 M urea, 0.02 M Bis-tris propane and 0.5% (v/v) 2-mecaptoethanol. The diluted sample was held at ~21°C for 1 h, shaken occasionally and filtered through a 0.2 μm filter (Nalgene®, Thermo Fisher Scientific, Pittsburgh, PA, USA) prior to loading onto the HPLC unit (RP-HPLC, Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA). The sample (5 μL) was injected and proteins resolved using the 300 SB-C18 RP Poroshell column (Agilent Technologies, Santa Clara, CA, USA) at 35°C before detecting at 214 nm and 280 nm using the diode array detector. The hydrophilic mobile phase (A) consisted of a mixture of acetonitrile, triflouroacetic acid (TFA) and HPLC grade water (Milli-Q water; 18.2

MΩ cm) at a ratio of 100:1:900 (v:v:v) filtered through a hydrophobic filter paper (0.45 μm, Merck Millipore, MA, USA). The hydrophobic mobile phase (B), consisting of acetonitrile, triflouroacetic acid (TFA) and HPLC grade water (Milli-Q water; 18.2 MΩ cm) at the ratio of 900:1:100 (v:v:v), was filtered through hydrophilic filter paper (Merck Millipore, MA, USA). The flow rate was 0.5 mL/min. Peak areas were integrated manually and the concentration of individual proteins was calculated using the software with the aid of calibration curves for individual milk protein fractions (HPLC ChemStation Software, Agilent Technologies, Santa Clara, CA, USA).

2.2.7. Physico-chemical characteristics of skim milk samples

The size of casein micelles in the milks was determined by dynamic light scattering using the Malvern Zetasizer Nanoseries Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK). The milk sample was diluted 1:10 (v/v) in simulated milk ultrafiltrate (SMUF) prepared according to Jenness and Koops (1962) and mixed well for 30 s. The measurement was made with a backscatter angle of 173° at 25°C in DTS0012 disposable sizing cuvettes

The apparent zeta potential is a measure of the net charge on the protein particle and is determined on the basis of its movement (direction and velocity) in an applied electric field. The apparent zeta potential of the diluted samples was also measured using the Malvern Zetasizer Nanoseries Nano-ZS equipped with a 2 mW Helium-Neon laser with an output of 633 nm, as described by O'Kennedy and Mounsey (2009). The diluted milk sample (~ 21°C) was injected into an equilibration chamber and held for 2 min at 25°C. The sample was then subjected to an applied potential of 150 V at a modulation frequency of 250 Hz.

The pellet obtained on ultracentrifugation of milk was weighed following decanting of the serum phase, frozen at -80°C and lyophilized at -46°C (FreeZone Freeze Dry Systems, Labconco, Kansas City, MO, USA) under vacuum ($\leq 130 \times 10^{-3}$ mBar). The difference in the weight of the wet pellet and lyophilized pellet was taken as the water bound to casein micelles, and expressed as g water/g casein (Creamer, 1985).

2.2.8. Rennet gelation

Skim milk was adjusted to pH 6.55 at room temperature; the temperature of the pH-adjusted skim milk was adjusted to 31°C and rennet (Chy-Max® plus, 200 IMCU/mL; Chr. Hansen, Hørsholm, Denmark), diluted 1:20 in distilled water, was added on a protein basis at a level equivalent to 5.1 mL of undiluted enzyme per kilogram of milk protein. A 10 ml sample was pipetted into the rheometer cell, concentric cylinder geometry (with a 12 mm gap between the cylinders, 15 mm stator inner radius, 13.83 mm rotor radius, and 32 mm cylinder immersed height). The changes in storage modulus, G', which was used as an index of gel firmness, was measured dynamically as a function of time over 1 h at a strain of 0.025, which is less than the fracture strain (~3) of rennet gel aged for 1 h (Walstra, 1993), and a frequency of 1 Hz using dynamic low amplitude strain oscillation rheometry in a controlled stress rheometer (Carri-Med, CSL^2_{500}, TA Instruments, New Castle, DE, USA). The following parameters were calculated from the resultant G′/time curve: rennet gelation time (RGT), the time required for G' to increase to a value of ≥0.2 Pa; $G'_{60,}$ the value of G' at 60 min, and index of gel firmness or strength; and maximum gel firming rate (GFR_{max}), as the maximum slope of the G'/time curve.

2.2.9. Heat stability

The stability of the milk protein to aggregation at 140°C as a function of milk pH was monitored by observing the time required for visual flocculation to occur, as described (O'Connell & Fox, 2003).

Milk samples were adjusted to different pH values in the range from 6.2 to 7.2 (0.1 pH unit increments) at 21°C using 0.1 N HCl or NaOH. A sub-sample (3.4 g) was placed in 4 mL heat-resistant tube (120 mm tube length, 10 mm outer radius, 7 mm inner radius; Hettich Benelux B.V., Geldermalsen, Netherlands) which was capped with a rubber bung and placed and secured in a metal rack. The loaded rack was placed in the temperature-controlled oil bath (Hettich ESP oilbaths; Hettich Benelux BV, Geldermalsen, Netherlands) at 140°C and rocked gently at a constant frequency (7 oscillations /min). The heat coagulation time (HCT) is defined as the time required for the formation of visual flocs of aggregated protein on the walls of tubes.

2.2.10. Ethanol stability

Sub-samples of skim milk were adjusted to pH values of 6.2, 6.4, 6.6, 6.8 or 7.0 and mixed with aqueous ethanol solutions, ranging from 30 to 98% (v/v), at a volume ratio of 1:2 based on a milk protein content of 3.3%, which gave a ratio of ethanol-to-protein of 59.4 mL/g protein when 98% aqueous ethanol solution was used (Horne & Muir, 1990). The volume of added aqueous ethanol solution increased with increasing protein content of milk to keep the constant ratio of ethanol-to-protein. The ethanol stability (ES) was defined as the lowest concentration of aqueous ethanol required to induce flocculation when the ethanol/milk mixture was mixed by vibration for 30 s (Whirlimixer™, Fisons, Holmes Chapel, UK).

2.2.11. Statistical analysis

The milk samples were blocked arbitrarily into four seasons, namely Spring (March, April and May), Summer (June, July and August), Autumn (September, October and November) and Winter (January and February) (Figure 2.1a). Analysis of variance (ANOVA) was carried out using a general linear model (GLM) procedure of SAS 9.3 (Institute, 2011)and the effects of season on each response variable was determined. Tukey's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

Simple linear regression was carried out to determine whether the relationships between measured parameters were significant. The level of significance was determined at $P < 0.05$ for all analyses, according to Students t-test.

2.3. Results and discussion

2.3.1. Skim milk composition

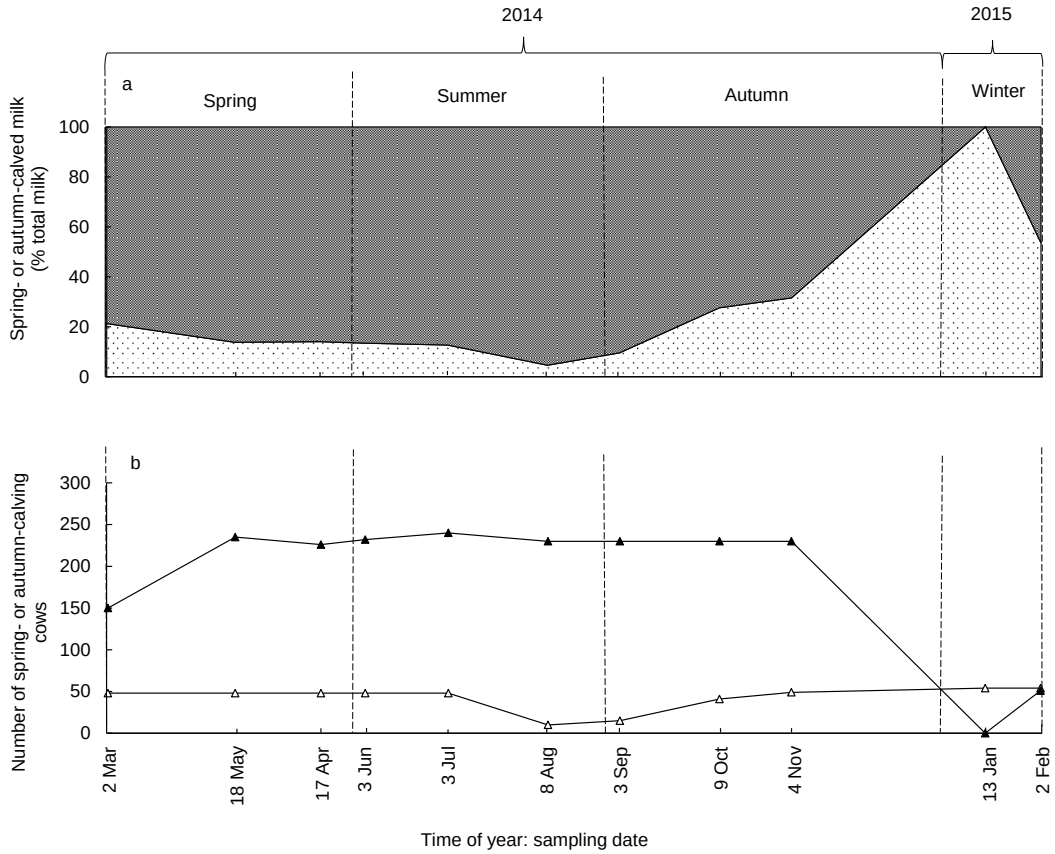

Figure 2.1. Seasonal variation in (a) proportions of milk from spring- (▩) or autumn- (⁘) calving-cows in a mixed herd, and (b) the number of spring- (▲) or autumn- (△) calving cows. The bulked herd milk was sampled on 11 different occasions during the months of March 2014 to November 2014 and January 2015 to February 2015, and the samples were assigned arbitrarily to Spring (March, April and May), Summer (June, July and August), Autumn (September, October and November) or Winter (January and February) seasons.

The mean compositional parameters of the skim milk over the year are shown in Table 2.2. Despite variations throughout the year, season did not significantly affect the concentration of total solids, lactose, protein, casein, whey protein and NPN, or pH (6.63-6.75, data not shown). Similarly, Chen et al. (2014) reported that season did not significantly affect the mean concentrations of total solids, lactose or urea (major component of NPN) in bulk milk from a year-round calving herd in the

UK. However, in contrast to the current study, Chen et al. (2014) found that season had a significant effect on pH, which varied from 6.73 to 6.87, and the concentrations of protein and casein, which varied from 2.89 to 3.56 and 2.08 to 2.52%, respectively, compared to 3.34-3.94 and 2.61-3.02%, respectively, in the current study. Nevertheless, the percentage change (difference between maximum and minimum values, expressed as a % of the minimum) for concentrations of protein (18%), casein (15%) and whey protein (44%) over the season was comparable to those reported by Chen et al. (2014), but narrower than those (23, 25 and 53%, respectively) reported by O'Brien et al. (1999a) for manufacturing milk in Ireland over the course of a 12 month period. The percentage change over the year in lactose (9%) and NPN (~40%) in the current study were also similar to those reported by O'Brien et al. (1999b), but the percentage change in Ca throughout the year was higher (37%, *vs*. 24%).

Table 2.1. Seasonal variation in the proportions of milk from spring- and autumn-calved cows in a mixed herd, and the gross composition of raw milk[1]

Item[2,3]	Mean±SD	Range	Season			
			Spring	Summer	Autumn	Winter
Spring-calved milk (% total milk)	72.5±27.6	0-95.4	83.6[a]	89.8[a]	77.1[a]	23.2[b]
Autumn-calved milk (% total milk)	27.5±27.6	4.61-100	16.4[b]	10.2[b]	22.9[b]	76.8[a]
Composition						
Total solids (%, w/w)	13.2±0.52	12.1-14.0	13.0[a]	13.3[a]	13.4[a]	13.3[a]
Lactose (%, w/w)	4.92±0.1	4.70-5.05	4.90[a]	4.94[a]	4.92[a]	4.89[a]
Fat (%, w/w)	4.05±0.3	3.59-4.71	3.83[a]	4.03[a]	4.38[a]	3.94[a]
Protein (%, w/w)	3.55±0.15	3.39-3.90	3.45[a]	3.53[a]	3.69[a]	3.56[a]

[1]The bulked herd milk was sampled on 11 different occasions during the months of March 2014 to November 2014 and January 2015 to February 2015; the changes in proportions of milk from spring- and autumn-calving cows is shown in Figure 2.1.
[2]Values within a row not sharing a common superscript differ significantly ($P < 0.05$).
[3]SD, standard deviation.

Table 2.2. Seasonal variation in the composition and physico-chemical characteristics of skim milk from a mixed herd comprised of spring-and autumn-calving cows[1,2,3]

			Season			
	Mean±SD	Range	Spring	Summer	Autumn	Winter
Composition						
Total solids (%, w/w)	9.62±0.29	9.05 - 9.94	9.69[a]	9.42[a]	9.67[a]	9.81[a]
Lactose (%, w/w)	4.89±0.1	4.67 - 5.08	4.85[a]	4.88[a]	5.00[a]	4.82[a]
Protein (%, w/w)	3.58±0.17	3.34 - 3.94	3.46[a]	3.57[a]	3.70[a]	3.61[a]
Casein (%, w/w)	2.80±0.13	2.61 - 3.02	2.71[a]	2.82[a]	2.87[a]	2.82[a]
Individual caseins (% milk casein)						
α_{s1}-casein	41.1±2.0	37.8 - 44.9	40.4[b]	40.3[b]	43.4[a]	40.1[b]
α_{s2}-casein	8.21±1.30	7.04 - 11.64	7.68[a]	7.84[a]	9.11[a]	8.38[a]
β-casein	36.6±3.1	29.8 - 40.7	38.6[a]	37.8[ab]	33.1[b]	36.2[ab]
κ-casein	14.1±1.4	11.5 - 16.7	13.3[a]	14.0[a]	14.4[a]	15.3[a]
Whey protein （% TP）	15.8±1.0	14.1 - 17.5	15.8[a]	15.4[a]	15.8[a]	16.4[a]
NPN (% total N)	5.88±0.72	4.87 - 7.61	6.03[a]	5.62[a]	6.45[a]	5.30[a]
Ca (mg/100 g)	123±12	104 - 142	112[a]	128[a]	131[a]	118[a]
P (mg/100 g)	101±8	89 - 111	103[ab]	94[b]	100[ab]	110[a]
Physico-chemical characteristics						
Casein micelle size (nm)	159±3	155 - 163	156[b]	160[a]	161[a]	161[a]
Zeta potential (mV)	–21.9±1.0	–20.6 - –23.9	-21.0[b]	-22.9[a]	-21.8[ab]	-21.4[b]
Casein hydration (g water/ g casein)	2.85±0.12	3.03 - 3.25	3.07[a]	2.95[a]	3.02[a]	3.14[a]

[1]The bulked herd milk was sampled on 11 different occasions during the months of March 2014 to November 2014 and January 2015 to February 2015.
[2]Values within a row not sharing a common superscript differ significantly ($P < 0.05$)
[3]SD, standard deviation; TP, total protein; NPN, non-protein nitrogen; Ca, calcium; P, phosphorus

The mean levels of α_{s1}-, α_{s2}-, β- and κ-caseins over the season, i.e., ~41.0, 8.2, 36.6 and 14.1% of total casein respectively, were typical of those reported in the literature for bovine milk (Bernabucci et al., 2015). Season affected the proportions of α_{s1}- and β-caseins significantly, with autumn milk having a significantly higher proportion of α_{s1}-casein than milk from Spring, Summer or Winter, and a lower proportion of β-casein than spring milk. This trend differs from that reported by

Donnelly and Barry (1983) for Irish manufacturing milk, which showed a progressive decrease in the proportion of α_s-casein from January to December and β-casein from June to December.

Ca content varied from 104 to 142 mg/100 g across the year but was not influenced significantly by season. The yearly variation, though wide, is consistent with that reported previously for spring-calved or autumn-calved bulk herd milks, e.g., 109-123 mg/100 g in bulk herd 'liquid' milk from a mixed calving herd in Ireland (Kelly et al.,1982), 98-126 mg/100 g for bulk herd milk from a year round-calving herd in the UK (Chen et al., 2014), 115-131 mg/100 g for a spring-calving herd milk (White & Davies, 1958a), and 100-142 mg/100 g (Kelly et al., 1982) or 108-138 mg/100 g (O'Brien et al., 1999b) for Irish manufacturing milks. P content varied also with season (89-111 mg/100 g) and was influenced significantly by season, being significantly lower in summer milk than in winter milk. The seasonal variation in P was comparable to that (80 to 102 mg/100 g) reported by White & Davies (1958a), but larger than that (83 to 95 mg/100 g) obtained for bulk milk samples from 9 different dairy plants in Sweden (Lindmark-Månsson et al., 2003).

2.3.2. Physico-chemical characteristics of skim milk

While casein micelle size in spring milk was slightly, but significantly, lower than that of milks from the other seasons, the overall seasonal variation in size was quite small (154–163 nm). Seasonal variation in casein micelle size has also been reported by others, including Holt and Muir (1978) and Glantz et al. (2010). The former study showed that the casein micelle size of creamery milk in Scotland was significantly lower in Summer (June-August; ~135 nm) than in Winter (December-February; ~170 nm), while the latter study found a significantly lower casein micelle size in summer milk (179 nm) than in winter (204 nm).

ζ-Potential of milk at natural pH is an index of the net negative charge on the casein micelles and, hence, reflects the extent of inter-micellar electrostatic repulsion and stability in the milk. The current values (-20.6 to -23.9 mV) are within the range reported previously for bovine milk at natural pH, i.e., -18.2 to -28 mV (Glantz et al., 2010; Grimley, et al., 2009). The ζ-potential of summer milk (-22.9 mV) was slightly, but significantly, higher than that of spring milk or winter milk (~ -21.0 mV) in the current study.

Casein micelle hydration is the quantity of water entrapped within the micelle and reflects both the structure and extent of interaction between the caseins. Hence, factors that promote casein interaction, e.g., addition of divalent salts such as $CaCl_2$ (Sood et al., 1979; van Hooydonk et al., 1986), reduce hydration of casein in milk. Casein hydration varied over the year (2.8-3.3 g water/g casein) but was not significantly affected by season. The mean value of hydration at 25°C (3.04 ± 0.12 g water/g casein) was within the range reported in the literature (O'Connell & Fox, 2000).

2.3.3. Composition of skim milk serum

The mean value for serum N, expressed as protein, was 0.99 ± 0.08% (w/w), equivalent ~25.7–29.8% of total protein in milk (Table 2.3). Whey protein, casein and NPN accounted for ~63.0, 16.3 and 20.6% of total serum protein, respectively. Serum casein, as a % of milk casein, varied from 3.62–10.54% which was comparable in magnitude to that reported for fresh milk (5-10%) (Dalgleish & Law, 1988). While season did not influence the concentrations of total protein, casein or whey protein in the serum, it had significant effect on the proportion of α_s (α_{s1} + α_{s2})- and κ-caseins. α_s-Casein, as a proportion of serum casein, varied from 19.3 to

48.3% across the year, being lowest in Spring (23.8%) and highest in Autumn (37.8%), while κ-casein, which varied from 11.3 to 45.4% of serum casein, showed an opposite trend with season.

While the concentration of Ca in the serum was not influenced by season, serum Ca as a proportion of total Ca was significantly higher in Summer than in Winter or Spring (Table 2.3). The mean concentration of serum P in winter-milk was higher than that of milk from Summer or Autumn. Serum P as a proportion of total P was significantly lowest in Autumn.

2.3.4. Rennet gelation

The rennet gelation characteristics (RGT, G'_{60}) of skim milk samples are shown in Table 2.4. Despite monthly variations, the mean values of RGT, GFR_{max} and G'_{60} were not affected significantly by season (Table 2.4). All rennet gelation parameters were dependent on milk casein content, as reflected by the significant positive correlation between milk casein content and GFR_{max} or G'_{60} and the inverse correlation between casein and RGT (Table 2.5). The trend is consistent with findings from previous studies (Guinee et al., 1996; Malacarne, et al., 2014), which found a power law relationship between milk protein (and hence casein) content and gel firmness or gel-firming rate (Guinee et al., 1997). The strong relationship between casein level and rennet gelation is expected as the strength of the gel is proportional to the volume fraction of gel building material (calcium phosphate *para*-casein) (Walstra et al., 1985).

Table 2.3. Seasonal variation in the composition of the serum phase of skim milk from a mixed herd comprised of spring-and autumn-calving cows[1,2,]

			Season			
Item[3,4]	Mean±SD	Range	Spring	Summer	Autumn	Winter
Protein (%, w/w)	0.99±0.08	0.90 - 1.15	0.93[a]	0.98[a]	1.05[a]	1.01[a]
Casein (%, w/w)	0.16±0.07	0.10 - 0.30	0.09[a]	0.20[a]	0.17[a]	0.18[a]
Casein (% total milk casein)	5.82±2.49	3.62 - 10.54	3.70[a]	7.15[a]	5.92[a]	6.21[a]
Casein (% serum protein)	16.3±6.5	6.80 - 29.3	9.71[a]	20.4[a]	16.0[a]	17.0[a]
Individual caseins (% serum casein)						
α_{s1}+α_{s2}-casein	30.2±7.4	19.3 - 48.3	23.8[b]	30.2[ab]	37.8[a]	28.6[ab]
β-casein	39.9±2.9	33.4 - 43.4	38.2[a]	40.4[a]	40.7[a]	40.4[a]
κ-casein	29.8±8.7	11.3 - 45.4	38.0[a]	29.4[ab]	21.5[b]	31.0[ab]
Whey protein (%, w/w)	0.62±0.08	0.62 - 0.78	0.55[a]	0.55[a]	0.59[a]	0.59[a]
Whey protein (% serum protein)	63.0±7.0	50.4 - 78.9	69.5[a]	58.7[a]	62.3[a]	63.3[a]
NPN (% serum N)	20.6±4.0	11.1 - 26.3	19.8[a]	20.9[a]	21.7[a]	19.7[a]
Ca (mg/100 g)	41±10	32 - 63	35[a]	49[a]	43[a]	33[a]
Ca (% total milk Ca)	33.2±5.2	27.2 - 45.3	31.6[b]	37.7[a]	32.3[ab]	28.0[b]
P (mg/100 g)	45±7	34 - 57	52[ab]	42[bc]	37[c]	54[a]
P (% total milk P)	45.1±5.8	33.6 - 51.2	50.2[a]	45.0[ab]	37.3[b]	49.4[a]

[1]The bulked herd milk was sampled on 11 different occasions during the months of March 2014 to November 2014 and January 2015 to February 2015.
[2]Skim milk serum was obtained on ultracentrifugation of milk at 100,000 *g* for 1 h at 25°C.
[3]Values within a row not sharing a common superscript differ significantly ($P < 0.05$)
[4]SD, standard deviation; NPN, non-protein nitrogen; Ca, calcium; P, phosphorus.

Table 2.4. Seasonal variation in processing characteristics of skim milk from a mixed herd comprised of spring-and autumn-calving cows[1,2]

			Season			
Item[3]	Mean±SD	Range	Spring	Summer	Autumn	Winter
Rennet gelation						
RGT (min)	19.7±1.9	16.5 - 22.6	20.5[a]	20.6[a]	19.0[a]	17.7[a]
GFR_{max} (Pa/min)	3.62±0.71	2.67 - 5.02	3.31[a]	3.40[a]	3.81[a]	4.24[a]
G'_{60} (Pa)	105±11	71 - 146	95[a]	98[a]	111[a]	127[a]
Heat coagulation time (min)						
HCT_{npH}	13.8±3.8	9.7 - 19.6	15.9[a]	11.4[a]	13.1[a]	16.1[a]
HCT_{max}	15.5±2.8	10.2 - 18.6	15.8[a]	15.3[a]	17.4[a]	12.7[a]
HCT_{min}	5.1±0.9	4.1 - 7.6	5.20[a]	5.57[a]	4.78[a]	4.46[a]
Ethanol stability (%, v/v)						
$ES_{6.2}$	39±1.3	38 - 42	39[a]	39[a]	38[a]	38[a]
$ES_{6.4}$	50±3.6	44 - 56	53[a]	50[ab]	47[b]	50[ab]
$ES_{6.6}$	74 ±7.7	60 - 80	80[a]	78[ab]	68[ab]	65[b]
$ES_{6.8}$	86±2.8	82 - 92	87[a]	86[a]	83[a]	85[a]
$ES_{7.0}$	86±2.3	88 - 96	94[a]	93[a]	90[b]	90[b]

[1]The bulked herd milk was sampled on 11 different occasions during the months of March 2014 to November 2014 and January 2015 to February 2015.
[2] Values within a row not sharing a common superscript differ significantly ($P < 0.05$).
[3]SD, standard deviation; RGT, rennet gelation time; GFR, gel firming rate; G'_{60}, storage modulus at 60 min after addition of rennet; HCT_{npH}, heat coagulation time at natural milk pH; HCT_{max} and HCT_{min} are the maximum and minimum heat coagulation times, respectively, of the HCT/pH (6.2 - 7.2) curve; $ES_{6.2}$, $ES_{6.4}$, $ES_{6.6}$, $ES_{6.8}$, and $ES_{7.0}$ correspond to ethanol stability of milk at pH 6.2, 6.4, 6.6, 6.8 and 7.0, respectively.

2.3.5. Heat coagulation time

All milk samples had a type A HCT/pH profile, with a maximum HCT at pH 6.6–6.7 (HCT_{max}) and a minimum at pH 6.8–7.1 (HCT_{min}). The HCT at natural pH (HCT_{npH}) and HCT_{max} are shown in Table 2.4. Despite month-to-month variations, season did not have a significant effect on HCT at different pH values (Table 2.4).

The HCT_{npH} (9.7 to 19.6 min; Table 2.4) was relatively low compared to that reported by (Holt et al., 1978) for creamery (silo) skim milk, i.e., 15 to 27 min over a 12 month period from January to December. This lower HCT_{npH} compared to that of (Holt et al., 1978) may be attributable to the higher concentrations of lactose (4.67-

5.08 *vs.* 4.63-4.82%) and total Ca (104-142 *vs.* 99-118 mg Ca/100 g), and lower pH (6.63-6.75 *vs.* 6.73-6.84) of the milk samples in the current study. During heating of milk at 140°C, lactose is thermally degraded to formic acid and other organic acids which reduce the pH and accelerate protein aggregation (Huppertz, 2016; van Boekel et al., 1989). In contrast to the current study and that of Holt et al. (1978), Kelly et al. (1982) found a much larger variation in HCT_{npH} of creamery milk (~5-80 min) and liquid milk (~40-80 min) at 130°C, where creamery milk (March year 1-March year 2) was from spring-calving herds, and liquid milk from mixed herds of spring- and autumn-calving cows. Kelly et al. (1982) found a significant positive correlation between HCT_{npH} and urea level, which varied from ~2.1 to 4.6% total N in creamery milk and 2.6-4.6% in the liquid milk.

HCT_{max}, which occurred at pH 6.6 to 6.7, varied from 10.2 to 18.6 min. Simple linear regression analysis (Table 2.5) indicated a significant ($P < 0.05$), though weak, positive correlation between HCT_{max} and level of NPN, which contains ~ 55% urea (Kelly et al., 1982; Mehra et al., 1999). The positive effect of NPN on HCT_{max}, which concurs with the results of previous studies (Fox et al., 1980; Muir & Sweetsur, 1977), has been attributed to the thermal degradation of urea to ammonia, and effect which reduces the extent of pH reduction during the heating of milk.

Table 2.5. Relationships between compositional parameters and processing characteristics of skim milk from a mixed herd comprised of spring-and autumn-calving cows[1,2,3]

Simple linear regression	Correlation coefficient (r)
Rennet gelation characteristics	
RGT: casein (%, w/w)	–0.577*
GFR_{max}: casein (%, w/w)	+0.701**
G'_{60}: casein (%, w/w)	+0.683*
Heat coagulation time (min)	
HCT_{npH}: lactose (%, w/w)	–0.665*
HCT_{npH}: Ca (mg/100 g)	–0.758**
HCT_{npH}: serum Ca (mg/100 g)	–0.611*
HCT_{max}: NPN(% total N)	+0.642*
HCT_{max}: α_{s1}-casein (% total casein)	+0.658*
Ethanol stability	
$ES_{6.4}$: casein micelle size (nm)	–0.690*
$ES_{6.4}$: Ca (mg/100 g)	–0.693*
$ES_{6.4}$: serum P (mg/100 g)	+0.628*
$ES_{6.4}$: sedimentable Ca (mg/100 g)	–0.597*
$ES_{6.6}$: sedimentable Ca (mg/100 g)	–0.595*
$ES_{6.6}$: sedimentable P (mg/100 g)	–0.768**
$ES_{7.0}$: sedimentable P (mg/100 g)	–0.595*

[1]Correlations were obtained using simple linear regression analysis of the entire data set; only relationships found to be statistically significant are shown: **, $P < 0.01$; *, $P < 0.05$.
[2]Positive and negative correlations between two parameters are indicated by a positive sign (+) and a negative sign (–), respectively
[3]RGT, rennet gelation time; GFR, gel firming rate; G'_{60}, gel firmness at 60 min after addition of rennet; HCT_{npH}, heat coagulation time at the natural skim milk pH; HCT_{max} and HCT_{min} are the maximum and minimum heat coagulation times, respectively, of the HCT /pH (6.2- 7.2) curve; $ES_{6.4}$, $ES_{6.6}$ and $ES_{7.0}$, ethanol stability at pH 6.4, 6.6 and 7.0, respectively; TS, total solid; TP, total protein; NPN, non-protein nitrogen; Ca, calcium; P, phosphorus.

2.3.6. Ethanol stability

The ES increased significantly with pH for all milk samples, from 38-42% ethanol at pH 6.2 (Table 2.4) to 88-96% at pH 7.0 (Figure 2.2). The increase in ES with pH is consistent with previous studies (Horne & Muir, 1990; Horne & Parker, 1981) and is attributed to the increase in the net negative charge of the casein

micelles (Mohammed & Fox, 1986). Simple linear regression analysis indicated that ES at different pH values was correlated with different compositional parameters (Table 2.5).

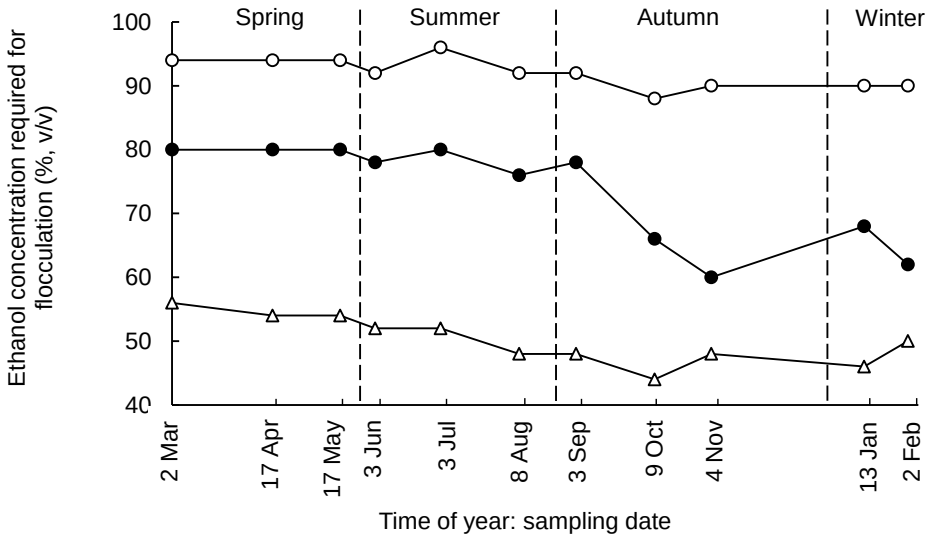

Figure 2.2. Seasonal variations in ethanol stability at pH of 6.4 (△), 6.6 (●) and 7.0 (○) of skim milk from a mixed herd comprised of spring-and autumn-calving cows as a function of time of year

ES at pH 6.6 ($ES_{6.6}$), which showed the largest variation with season (Table 2.4), correlated negatively with concentrations of sedimentable Ca and P (Table 2.5). The ES of winter milk at pH 6.6 was significantly lower than that of spring milk (Table 2.4); a similar trend was noted for ES at pH 7.0 ($ES_{7.0}$). ES at pH 6.4 ($ES_{6.4}$) was lower in autumn milk than in spring milk. ES at pH 6.4 correlated negatively with casein micelle size and total Ca content, and positively with concentration of serum P (Table 2.5). Similar trends were reported by Chen et al. (2014) who found an inverse relationship between casein micelle size and ES at natural pH (6.73-6.87), and Davies and White (1958) who found that the ES of late lactation, bulk herd milk at natural pH increased from 66 to 90% (v/v) ethanol as soluble inorganic P increased from 5 to 37 mg/100 mL.

2.4. Conclusions

The seasonal changes in the compositional, physicochemical and processability characteristics of skim milk from a mixed-herd with varying proportions of milk from spring- and autumn-calving cows was investigated during 2014-2015. The data were blocked according to season, denoted arbitrarily as Spring, March-May; Summer, June-August; Autumn, September-November; and Winter, January-February. Autumn milk, as a % of total milk, varied from ~10-20, 5-13, 20-40 and 50-100, in Spring, Summer, Autumn and Winter, respectively. Season affected concentrations of total P and serum P, levels of α_{s1}- and β-caseins (as proportions of total casein), casein micelle size, zeta potential, and ethanol stability at different pH values, significantly. While season did not influence the rennet gelation or heat stability characteristics of the milk, ethanol stability of autumn milk or winter milk at pH 7.0 was lower than that of spring- or summer-milk. The absence of significant seasonal effects on most compositional parameters, rennet gelation and heat-stability characteristics suggest that milk from a mixed-herd of spring- and autumn-calving cows is suitable for the manufacture of cheese and milk powder on a year-round basis, when the volume proportion of autumn milk, as a % of total, is similar to that of the current study. Hence, where milk is predominantly from a pasture-based, spring-calving system, as in Ireland, the use of autumn-calving herds at critical times of year (mid-October to February) can help to reduce the processability problems frequently encountered at the extremes of lactation.

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Chapter 3

Fortification of milk protein content with different dairy protein powders alters its compositional, rennet gelation, heat stability and ethanol stability characteristics

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Abstract

Skim milk (SM) was fortified from 3.3 to 4.1% protein using different milk protein powders: skim milk powder (SMP), native phosphocasein (NPC), calcium-reduced phosphocasein (CaRPC), sodium caseinate (NaCas) or calcium caseinate (CaCas). Compared to SMP or NPC, fortification with NaCas and CaRPC and to a lesser extent CaCas resulted in milk samples having higher proportions of non-sedimentable casein and calcium, and lower and higher levels of κ- and α_{s1}-casein, respectively, as a proportion of non-sedimentable casein. These changes coincided in milk samples fortified with NaCas, CaRPC or CaCas failing to undergo rennet-induced gelation, and having higher heat stability in the region 6.7-7.2 and ethanol stability at pH 6.4. The study demonstrates that the aggregation behaviour of protein-fortified milk samples is strongly influenced by the degree of mineralization of the protein powder used in fortification, which affects the partitioning of casein and calcium in the sedimentable and non-sedimentable phases.

Key words: Sodium caseinate, milk fortification, rennet gelation, heat stability, ethanol stability

3.1. Introduction

Milk protein powders find ubiquitous use as ingredients in the manufacture and stabilization of formulated foods and beverages, where they provide nutritional and/or techno-functional properties (e.g., water binding, viscosity, surface activity, gelation, structuring, heat stability). Applications include beverages such as cream liqueurs, infant milk formula and high protein supplements for sports performance and health nutrition (e.g., meal replacement) (Agarwal et al., 2015; Lagrange et al., 2015; O'Kennedy, 2009). They are also used in the preparation of recombined milk, protein-fortified milk or reassembled 'milk' that is converted into products such as cheese and yoghurt (Augustin, 1999; Guinee et al., 2009).

In some of the above applications, controlled protein aggregation is desirable while in others it is not. The stability of dairy proteins to high temperatures is critical in the preparation and stability of various foods and beverages including ultra-high temperature (UHT) treated milk, infant milk formula, coffee whiteners, cheese cake filling, confections, cooked meats, sauces, dairy desserts (Inglett & Inglett, 1982). The resistance of proteins to heat-induced aggregation minimises the occurrence of defects such as precipitation, flocculation and sandiness (Piorkowski & McClements, 2014). Despite the fact that dairy protein powders are frequently added to milk (or reconstituted milk) in the preparation of various foods and beverages, there is relatively little published information on the effect the type protein ingredient used on the heat- or ethanol-stability (Horne, 2003; Huppertz, 2016).

Controlled destabilisation and gelation of milk protein is a prerequisite for the manufacture of cheese and fermented milk products. Recombined milk or fortified milk can be formulated with different milk proteins for conversion into cheese that, from a regulatory perspective, may be made by coagulation of milk and/or products obtained from milk (FAO/WHO, 2011). Recombined milk is increasingly used in the manufacture of non-standard of identity cheeses for use as ingredients in the food-service and ready-meals sectors; a non-standard of identity cheese product is one which does not have a specific national standard but complies with the generic standard for cheese, as defined by the Codex Alimentarius (FAO/WHO, 2011). Nevertheless, there is, surprisingly, little information on the rennet coagulation characteristics of milk protein dispersions or milk fortified with different milk proteins such as calcium caseinate. Gaygadzhiev et al. (2012) reported the failure of skim milk to undergo rennet gelation when fortified with 0.2% (w/w) sodium caseinate; the addition of caseinate increased serum casein levels and reduced the extent of casein micelle aggregation. Nair and Corredig (2015) reported that the addition of 0.6% sodium caseinate to milk concentrated 3-fold had no effect on rennet-induced gelation when the milk was concentrated by ultrafiltration (as reflected by the similar magnitude of storage modulus in the concentrated milk with or without added sodium caseinate) but severely impeded gelation when the milk was concentrated by osmotic concentration using polyethylene glycol. The authors concluded that the dependence of the effect of sodium caseinate on the method of concentration may relate to potential differences in the

extent of rearrangement of the native micelles and the self-assembly of the added caseinate into micelles (Pitkowski et al., 2009).

The current study investigated the effect of type of milk protein ingredient used to fortify the protein content of milk on composition, rennet gelation, and stability to ethanol and heat.

3.2. Materials and methods

3.2.1. Ingredients used for fortification of milk

Extra low-heat skim milk powder (SMP) was manufactured using a pilot-scale NIRO Tall-Form Dryer in Moorepark Technology Limited (Cork, Ireland). Milk was separated to <0.1% fat, and the skim milk was treated at 72°C for 15 s, evaporated to 48-50% total solids, and spray dried using nozzle atomization at inlet and outlet air temperatures of 185°C and 85°C, respectively. The whey protein nitrogen index (WPNI) of the resultant low heat skim milk powder was 3.44 mg WPN/g. Native phosphocasein powder (NPC) and calcium-reduced phosphocasein powder (CaRPC) were also manufactured using microfiltration and diafiltration in the pilot plant at Moorepark Technology Limited, according to the method previously described by Guinee et al. (2009). The calcium to casein ratio was reduced from 33 mg Ca/g casein in NPC to 11 mg Ca/g casein in CaRPC by addition of trisodium citrate and citric acid to skim milk prior to microfiltration. Sodium caseinate (NaCas) and calcium caseinate (CaCas) were kindly donated by Kerry Ingredients Limited (Listowel, Ireland). The ingredients were analysed for protein, casein, whey protein, fat, lactose, non-protein nitrogen (NPN) and

minerals using the methods discussed below for analysis of fortified milk samples. The compositions of the ingredients are shown in Table 3.1.

3.2.2. Preparation of protein-fortified milk samples

Skim milk (SM) was prepared by dispersing SMP in distilled water at 50°C using high-shear mixing (6300 rpm for 5 min; Silverson model L4RT, Silverson, Chesham, UK) to give a final protein content of 3.3%. The skim milk was then stirred at 750 rpm for 10 min (Model RW16; IKA-Werke GmbH, Staufen im Breisgau, Germany). The appropriate quantity of milk protein ingredient was then added to SM and the blend was stirred for 20 min at 50°C before placing at 4°C for 22 h. Skim milk samples fortified with SMP, NPC, CaCas, CaRPC or NaCas were denoted as SM-SMP, SM-NPC, SM-CaCas, SM-CaRPC and SM-NaCas, respectively. For all assays, fortified milk samples were warmed at 40°C for 30 min to reverse the cold-ageing effects of overnight storage at 4°C (Dalgleish & Law, 1988; Fox, 1969).

3.2.3. Preparation of milk supernatant

Fortified milk samples were ultracentrifuged at 100,000 g at 25°C for 1 h using a fixed-angle Sorvall Titanium-1270 rotor (Sorvall Discovery 90SE ultracentrifuge, Kendro Laboratory Products, Asheville, NC, USA) (Dalgleish & Law, 1988). The resulting supernatants were filtered through glass-wool to remove any fat.

3.2.4. Compositional analysis of milk and supernatant

Milk samples were analysed for fat and total solids using the CEM SMART Trac II (CEM, North Carolina, USA), total protein by Kjeldahl (International Dairy Federation, IDF, 2001a) and lactose using the FOSS

MilkoScan™ FT+ (Foss Electric A/S, Hillerød, Denmark). Samples of milk and supernatant were analysed for Ca and P using atomic absorption spectrometry and spectrophotometer, respectively (Chapter 2). Total nitrogen (N), non-casein nitrogen (NCN) and the NPN of milk samples and the corresponding supernatant were determined according to standard IDF methods (IDF, 2001a, b). The concentration of ionic calcium [Ca^{2+}] was measured using a sensION+ 9660C Calcium Combination Ion Selective Electrode (Hach Lange, Barcelona, Spain). Potassium chloride (3M) was added to the milk at a level of 1% (v/v) and stirred and the Ca^{2+} concentration was measured immediately while stirring; the electrode was calibrated using calcium chloride solutions varying in [Ca^{2+}] (0.5 – 5 mM).

The individual proteins in milk and supernatant were analysed by reversed-phase high pressure liquid chromatography (RP-HPL; Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA) using a 300 SB-C18 RP Poroshell column (Agilent Technologies, Santa Clara, CA, USA), according to the method of Visser et al. (1991).

3.2.5. Casein micelle characteristics

Casein micelle size was determined in triplicate by dynamic light scattering using the Malvern Zetasizer Nanoseries Nano-ZS (Malvern Instruments Ltd, Malvern, UK). The milk sample was diluted 1:10 (v/v) in simulated milk ultrafiltrate (SMUF) prepared according to Jenness and Koops (1962). The measurement was made with a backscatter angle of 173° at 25°C in DTS0012 disposable sizing cuvettes, and the result presented as the number mean (Maher et al., 2011). The apparent zeta potential of the diluted samples was also measured using the Malvern

Zetasizer Nanoseries Nano-ZS equipped with a 2 mW Helium-Neon laser with an output of 633 nm, as described by O'Kennedy and Mounsey (2009).

Casein micelle hydration was calculated as the moisture: casein ratio (g water/g casein) of the pellet obtained on ultracentrifugation, as described in Chapter 2. Casein in the pellet was calculated as the difference in casein content between the milk and its supernatant.

3.2.6. Rennet gelation

The rennet gelation properties of milk samples were measured in triplicate at pH 6.55 and 31°C using low amplitude strain oscillation rheometry (LASOR) in a controlled stress rheometer (Carri-Med, type CSL^2_{500}, TA instruments, New Castle, DE, USA), as described by Guinee et al. (2006). Chymosin (single strength Chy-Max® plus, 200 IMCU/mL; Chr. Hansen, Hørsholm, Denmark), diluted 20-fold with distilled water, was added at a level of 421 μl/100 mL milk sample. The storage modulus, G' was measured dynamically as a function of time over 1 h at a strain of 0.025 and a frequency of 1 Hz as described in Chapter 2.

3.2.7. Proteolysis during renneting

Milk fortified with SMP or NaCas was adjusted to pH 6.55 and tempered to 31°C. Chymosin was added at level of 421 μl 100mL and mixed in over a period of 30 s. The rennet-treated milk was then incubated quiescently for 60 min in a thermostatically controlled water bath (Grant Instruments, Cambridge, UK). The level of proteolysis of κ-casein was determined by measuring the change in the level of N soluble in 12% TCA at different time intervals during the 60-min incubation period (Hindle & Wheelock, 1970). A sample blank was prepared by adding distilled water instead of chymosin to the milk and incubating as described. The TCA digests

were filtered through No. 42 filter paper (WhatmanTM 1442-50l; GE Healthcare Life Sciences, Little Chalfont, UK), and the total nitrogen content determined by Kjeldahl (IDF, 2001a).

3.2.8. Heat stability

Milk samples were adjusted to different pH values in the range from 6.2 to 7.2 (0.1 pH unit increment) at room temperature using 0.1 N HCl or NaOH. The heat coagulation time at 140°C was measured as described by O'Connell and Fox (2000) and Chapter 2.

3.2.9. Ethanol stability

Milk samples were adjusted to different pH values in the range 6.2 to 7.0 (0.2 pH unit intervals). Ethanol stability was measured as described by Horne and Muir (1990), and involved blending of milk samples with aqueous ethanol solutions, ranging in concentration from 98 to 30 % (v/v) in 2% intervals, at a milk-to-ethanol volume ratio of 1:4.8. Following ethanol addition, the mixture was agitated by vibration for 30 s and inspected for visible signs of flocculation (Chapter 2). α-lactose monohydrate powder (> 99.0% lactose; Arla Foods Ingredients, Sønderhøj, Denmark) was added to NaCas-fortified milk samples at a level of 4.5, 4.9, 5.3 or 5.8%.

3.2.10. Statistical analysis

Data were analysed using a randomized complete block design, which incorporated treatment milk samples (fortified with different ingredients) and 4 blocks (batches of treatment milk made on different days). Analysis of variance was carried out using a general linear model (GLM) procedure of SAS 9.3 (SAS Institute, 2011) and the effects of treatment and replicate on each response variable was

determined. Tukey's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

3.3. Results and discussion

3.3.1. Composition of skim milk and fortified milk samples

The compositional characteristics of the fortified milk samples are shown in Table 3.2. Milk samples fortified with ingredients having a significantly lower level of total Ca- or P-to-protein ratio than SMP (e.g., NaCas, CaCas, CaRPC) had significantly lower levels of total Ca and P than the SM-SMP or SM-NPC milk samples. The [Ca^{2+}] was lowest in the SM-NaCas milk and highest in SM-CaCas milk; the lower [Ca^{2+}] in the SM-NaCas milk compared to SM-SMP indicates a reduction in the ionic calcium present in the serum phase of native skim milk owing to its association with the added NaCas, which had a low content of total calcium (Table 3.1). The fat content of all milk samples was < 0.1%, w/w (data not shown).

Table 3.1. Composition of dairy ingredients used for fortifying of the protein content of milk[1,2,3]

	Ingredients				
Composition	SMP	NPC	CaCas	CaRPC	NaCas
Total protein (%, w/w)	36.4^{e}	78.4^{c}	86.6^{b}	73.1^{d}	87.9^{a}
Casein (% TP)	82.8^{b}	93.7^{a}	98.6^{a}	96.4^{a}	97.0^{a}
Whey protein (% TP)	11.2^{a}	4.6^{b}	0.4^{c}	1.8^{c}	1.2^{c}
Fat (%, w/w)	0.73^{c}	0.84^{b}	0.63^{e}	1.43^{a}	0.67^{d}
Lactose (%, w/w)	46.35^{a}	2.12^{b}	1.09^{b}	1.90^{b}	1.27^{b}
Ca (mg/100 g)	1295^{b}	2395^{a}	1158^{c}	753^{d}	193^{e}
P (mg/100 g)	913^{b}	1275^{a}	761^{c}	608^{d}	687^{cd}

[1]Ingredient code: SMP, low heat skim milk powder; NPC, native phosphocasein; CaCas, calcium caseinate; CaRPC, calcium-reduced phosphocasein; NaCas, sodium caseinate

[2] Values within a row not sharing a common superscript differ significantly ($P < 0.05$)

[3]TP, total protein; Ca, calcium; P, phosphorus

Table 3.2. Compositional characteristics of protein-fortified milk samples[1,2]

	Fortified milk samples				
Composition[3]	SM-SMP	SM-NPC	SM-CaCas	SM-CaRPC	SM-NaCas
TP (%, w/w)	4.10^{a}	4.10^{a}	4.09^{a}	4.10^{a}	4.10^{a}
Casein (%, w/w)	3.12^{c}	3.27^{b}	3.34^{ab}	3.39^{a}	3.39^{a}
Whey protein (%, w/w)	0.73^{a}	0.59^{b}	0.55^{c}	0.65^{c}	0.54^{c}
NPN (% TP)	5.66^{a}	4.29^{b}	4.01^{b}	3.91^{b}	3.80^{b}
Lactose (%, w/w)	5.78^{a}	4.51^{b}	4.43^{b}	4.54^{b}	4.53^{b}
pH	6.70^{ab}	6.70^{ab}	6.66^{b}	6.71^{ab}	6.74^{a}
Ca (mg / 100g)	161^{a}	162^{a}	138^{b}	138^{b}	132^{b}
Ionic Ca (mM)	1.93^{ab}	1.87^{ab}	2.08^{a}	1.82^{bc}	1.61^{c}
P (mg /100 g)	116^{a}	109^{a}	98^{b}	97^{b}	96^{b}

[1]Sample code: Protein content of milk was fortified from 3.3 to 4.1% using skim milk powder (SM-SMP), native phosphocasein (SM-NPC), calcium caseinate (SM-CaCas), calcium-reduced phosphocasein (SM-CaRPC) or sodium caseinate (SM-NaCas).
[2]Presented values are the means of quadruplicate batches for each treatment; values within a row not sharing a common superscript differ significantly ($P < 0.05$).
[3]TP, total protein; NPN, non-protein nitrogen; TN, total nitrogen; Ca, calcium; P, phosphorous.

The RP-HPLC casein profile of all milk samples was typical of that previously reported for bovine milk (Pesic et al., 2012), with α_{s1}-, α_{s2}-, β-, and κ-caseins accounting for ~38.5, 9.7, 34.4 and 17.4% of total casein in the SM-SMP milk (data not shown). The levels of α-lactalbumin (α-Lac), β-lactoglobulin A (β-LgA) and B (β-LgB), as a % of total whey protein, were similar to those reported by Bordin et al. (2001) but higher than those given elsewhere (Fox, 2003), probably because the minor whey proteins, including bovine serum albumin (BSA), lactoferrin and immunoglobulins were not detected by the RP-HPLC protocol used.

3.3.2. Particle size and zeta potential

All milk samples showed a mono-modal particle size/number distribution with a number mean varying from 125–142 nm (data not shown), typical of that reported previously for bovine milk (O'Connell & Fox, 2000). This trend concurs with that reported by Gaygadzhiev et al. (2012), who found that the addition of sodium caseinate (0.2%, w/w) to skim milk did not significantly alter the apparent diameter, as measured using dynamic light scattering. Similarly, Sandra and Corredig (2013) found that the addition of MPC to skim milk did not affect the surface weighted mean particle diameter (D_{32}) or volume weighted mean particle diameter (D_{43}), as measured using light scattering. The zeta potential of all milk samples was ~ -23 mV and did not significantly differ with ingredient used for fortification (data not shown). This value is similar to that reported for skim milk by Philippe et al. (2005), higher charge than that found in other studies for skim milk, e.g. -17.2 mV (O'Connell & Fox, 2000), and lower charge than that (-34 mV) reported by Sejersen et al. (2007). The inter-study differences may reflect differences in sample diluent (SMUF or deionized water) and measuring conditions, including milk temperature and pH, and the applied voltage.

3.3.3. Casein hydration

Casein hydration, expressed as g water/g sedimentable casein, was significantly affected by the type of ingredient used in fortification. The value for the SM-SMP milk (Table 3.3) was typical of that reported previously for native bovine skim milk (Creamer, 1985; O'Connell & Fox, 2000). Casein hydration in the SM-NPC milk was significantly lower than that of the SM-SMP and SM-NaCas milk samples but similar to that in the SM-CaCas and SM-CaRPC milk samples. A tentative explanation for the relatively lower casein micelle hydration in the SM-

NPC, compared to SM-SMP, is the low level of lactose in the NPC (Table 3.1). Baldwin (2010) suggested that lactose in skim milk powder assists in rehydration of the casein micelle, firstly reducing the extent of casein interaction during drying (by acting as a mechanical spacer between, and by hydrogen bonding to, the protein chain), and secondly by absorbing water during reconstitution. While the lactose content is also low in the NaCas, CaCas and RCaPC, the negative impact of low lactose content on hydration may have been mitigated by their lower calcium levels (Sood et al., 1979).

3.3.4. Composition of milk supernatants

The concentration of non-sedimentable protein, casein and P in the supernatants differed significantly with ingredient type used for fortification (Table 3.3). While the concentrations of non-sedimentable Ca (~58 mg/100 g) for all milk samples was similar, the level of supernatant Ca, as a proportion of total Ca, in SM-NaCas, SM-CaCas and SM-CaRPC was significantly higher than that in SM-SMP and SM-NPC.

The proportion of non-sedimentable casein in SM-SMP (6.1% of total casein) was similar to that reported for fresh skim milk (~5-10%) by Dalgleish and Law (1988) using similar ultracentrifugation conditions. The level in SM-CaRPC or SM-NaCas was significantly higher than that in the other milk samples, which did not differ significantly (Table 3.3). The high proportions of non-sedimentable casein and Ca in milk fortified with NaCas agrees with the results from previous studies involving the addition of sodium caseinate to skim milk (Gaygadzhiev et al., 2012; Parker et al., 2005), aqueous dispersions of phosphocasein (Thomar & Nicolai, 2015) or concentrated milk (Nair & Corredig, 2015). The higher level of non-sedimentable

casein in the SM-NaCas milk suggests that at least some of the added NaCas remained non-sedimentable when added to the skim milk.

Table 3.3. Composition of supernatant and pellet obtained on ultracentrifugation of protein-fortified milk samples. [1,2,3]

	Fortified milk samples				
Composition[4]	SM-SMP	SM-NPC	SM-CaCas	SM-CaRPC	SM-NaCas
Supernatant					
Protein (%, w/w)	1.15^{b}	0.95^{b}	1.14^{b}	1.41^{a}	1.37^{a}
Casein (% total casein)	6.1^{b}	4.2^{b}	8.5^{b}	18.9^{a}	17.9^{a}
Whey protein (% total whey protein)	95.7^{b}	93.5^{c}	100^{a}	100^{a}	93.3^{c}
Ca (mg/100 g)	58^{a}	55^{a}	56^{a}	56^{a}	57^{a}
Ca (% total Ca)	35.8^{b}	34.0^{b}	40.6^{a}	40.8^{a}	42.9^{a}
P (mg/100 g)	51^{a}	47^{ab}	45^{b}	46^{ab}	48^{ab}
P (% total P)	44.1^{ab}	42.8^{b}	45.3^{ab}	47.4^{ab}	49.2^{a}
Sedimented layer (pellet)					
Casein (%, w/w)	2.93^{a}	3.13^{a}	3.06^{a}	2.75^{b}	2.79^{b}
Casein (% milk casein)	93.9^{a}	95.8^{a}	91.5^{a}	81.1^{b}	82.1^{b}
Ca (mg/g casein)	35.4^{a}	34.1^{a}	26.7^{b}	29.8^{b}	27.2^{b}
P (mg/g casein)	22.1^{a}	20.0^{ab}	17.6^{c}	18.6^{bc}	17.4^{c}
Molar ratio Ca:P	1.24^{ab}	1.32^{a}	1.18^{b}	1.24^{ab}	1.21^{b}
Casein hydration (g water /g sedimentable casein)	3.07^{a}	2.61^{b}	3.03^{ab}	2.93^{ab}	3.23^{a}

[1]Milk supernatant was obtained by ultracentrifugation of milk samples at 100,000 *g* at 25°C for 1 h.
[2]Sample code: Protein content of milk was fortified from 3.3 to 4.1% using skim milk powder (SM-SMP), native phosphocasein (SM-NPC), calcium caseinate (SM-CaCas), calcium-reduced phosphocasein (SM-CaRPC) or sodium caseinate (SM-NaCas).
[3]Presented values are the means of quadruplicate batches of each treatment; values within a row not sharing a common superscript differ significantly ($P < 0.05$).
[4]Ca, calcium; P, phosphorous.

For all milk samples, the level of individual caseins in the supernatant, expressed as % of their corresponding level in milk, was highest for κ-casein and lowest for α_{s2}/α_{s1}-caseins (Figure 3.1a). A similar trend has been reported for the proportions of individual caseins dissociated from casein micelles on cooling or

acidifying milk in the pH range 6.7 to 4.8 (Dalgleish & Law, 1988). As for non-sedimentable casein, the proportions of individual caseins in the serum of the SM-NaCas and SM-CaRPC milk samples were significantly higher than those of the SM-SMP, SM-NPC and SM-CaCas milk samples, which had similar levels (Figure 3.1a).

When expressed as a % of total non-sedimentable casein, the levels of κ-and β-casein were higher than the corresponding levels of α_{s1}- and α_{s2}-caseins in all milk samples (Figure 3.1b). Compared to the other milk samples, the SM-NaCas and SM-CaRPC milk samples had a significantly lower proportion of non-sedimentable κ-casein and a higher proportion of α_{s1}-casein. This trend concurs with the results of Thomar and Nicolai (2015) who found that α_{s1}-casein, as a proportion of non-sedimentable casein, increased progressively with level of sodium caseinate added to an aqueous dispersion of native phosphocasein micelles.

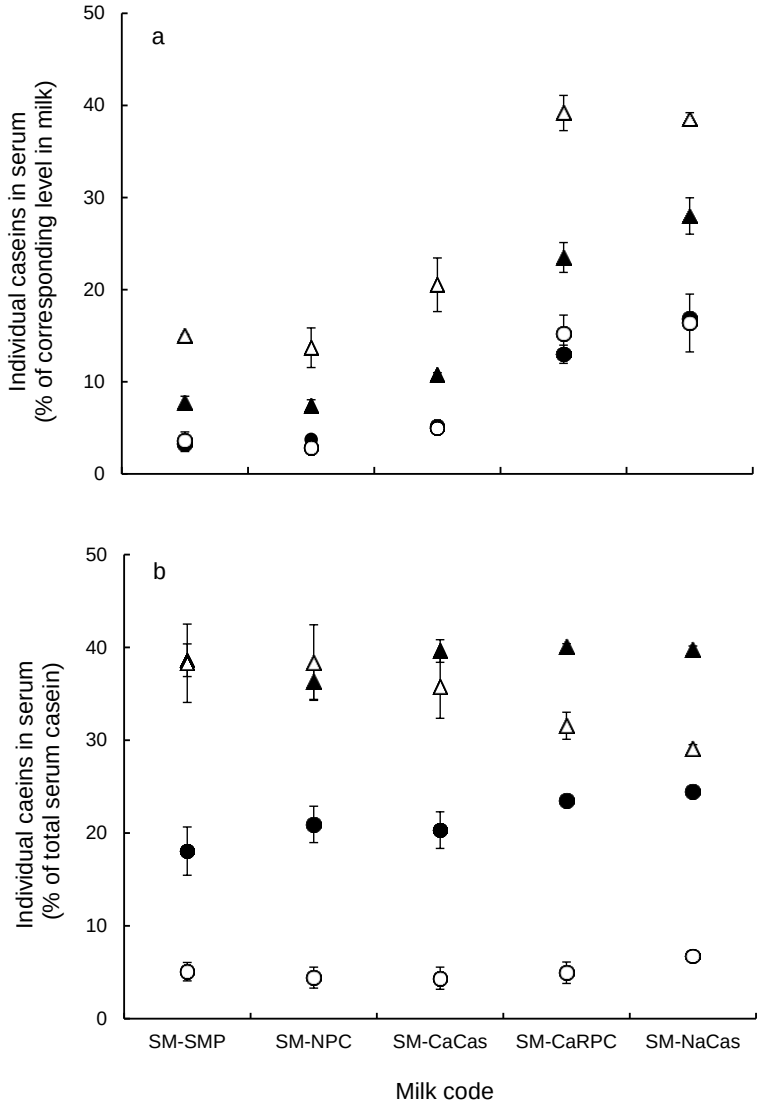

Figure 3.1. Individual caseins in the supernatants from fortified milk samples ultracentrifuged at 1000, 000 *g* at 25°C: α_{s1}- (●), α_{s2}- (○), β- (▲) and κ- (△) casein. Casein conentration is expressed as % of the corresponding concetration in milk (A) or % of total casein in the supernatant (B). The protein content of milk was fortified 3.3 to 4.1 % using skim milk powder (SM-SMP), native phosphocasein (SM-NPC), calcium caseinate (SM-CaCas), calicum-reduced phosphocasein (SM-CaRPC) or sodium caseinate (SM-NaCas). Presented values are the means of quadruplicate batches of each treatment milk; error bars represent standard deviation of the mean.

3.3.5. Rennet gelation properties

The change in G′ as a function of time from rennet addition for the different milk samples is shown in Figure 3.2 for Trial 1; similar G′/time profiles were obtained in the replicate trials (data not shown). The gel firming rate and G′60 of SM-NPC were significantly higher than that of SM-SMP. This trend concurs with previous results showing that milk standardized from 3.3 to 4.0% protein using NPC powder had higher G′ values than the corresponding milk samples standardized by addition of milk protein concentrate or by ultrafiltration (Guinee et al., 2006). This effect is expected because of the higher level of casein in the NPC powder compared to the SM-SMP, in which ~18% of the true protein is native whey protein that does not participate in rennet-induced gelation.

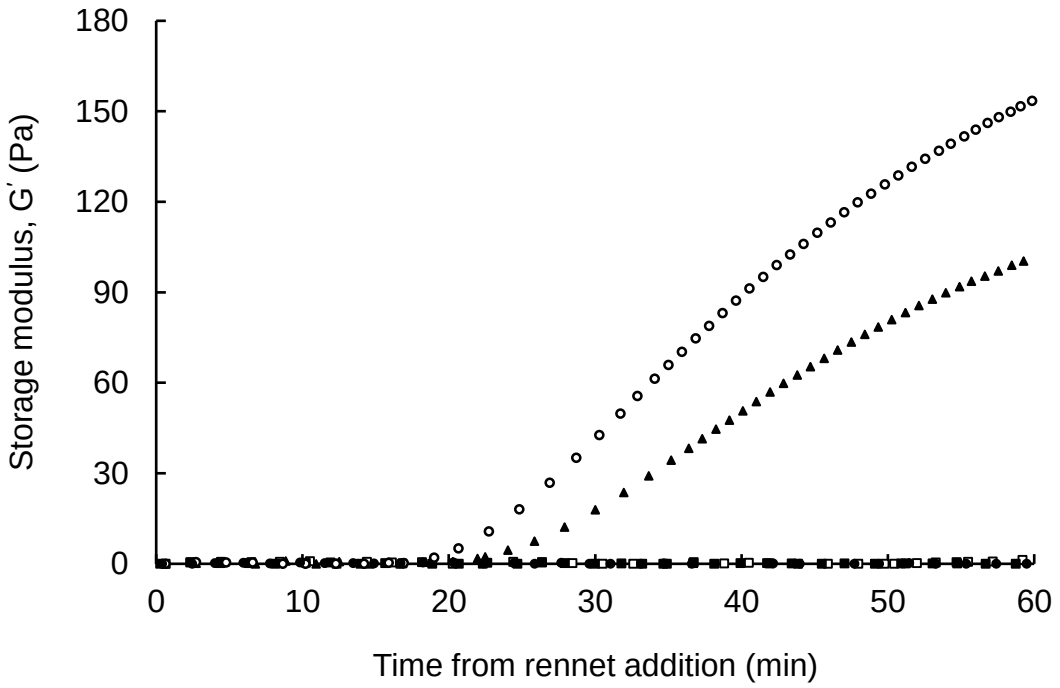

Figure 3.2. The developmment storage modulus, G′, in rennet-treated milk samples in which protein content was fortified from 3.3 to 4.1 % using different protein ingredients: skim milk powder, SMP (▲); native phosphocasein, NPC (○); calcium caseinate, CaCas (□); calcium-reduced phosphocasein, CaRPC (●) or sodium caseinate, NaCas (■).

Conversely, the addition of NaCas, CaCas or CaRPC resulted in failure of the fortified milk to coagulate in 60 min (Figure 3.2), despite the fact that the rennet-to-casein ratio, pH and temperature were standardized. A similar trend was reported by Gaygadzhiev et al. (2012), and Nair and Corredig (2015) who found that rennet gelation failed to occur on addition of NaCas at ≥0.2% (w/w). The results suggest that despite the similar levels of κ-casein hydrolysis (as discussed below), the failure of the latter milk samples to undergo rennet gelation may be associated with their significantly lower [Ca^{2+}] in the SM-NaCas milk and ratio of calcium-to-sedimentable casein in the SM-NaCas, SM-CaRPC and SM-CaCas milk samples (Table 2, 3) (Sandra et al., 2012; Singh, et al., 1988) and the higher proportion of non-sedimentable casein (in the SM-CaRPC and SM-NaCas milk samples). Analogously, reducing the total calcium content of milk or protein dispersions has been found to significantly impair rennet-induced gelation while increasing total calcium content has the opposite effect (Green, 1987; Sandra & Corredig, 2013). The impact of lower levels of ionic calcium and non-sedimentable calcium may be envisaged as a reduction in the degree of cross-linking of *para*-casein molecules. It is also feasible that the higher proportion of non-sedimentable casein in the SM-NaCas and SM-CaRPC may contribute to some type of steric impedance to aggregation of the *para*-casein micelles. Gaygadzhiev et al. (2012) hypothesized the adverse effect of NaCas on the rennet gel formation may be due to the adsorption of soluble casein on the surface of the micelles, with a consequential increase in the steric repulsion between the rennet-altered particles. Perhaps, serum casein hydrolyzed during renneting may also form into some kind of particles which impede the assembly of *para*-casein micelles into aggregates and, eventually, a gel.

The formation of N soluble in 12% TCA (12% TCA-SN) has been used as index of the degree of κ-casein hydrolysis by the coagulant during rennet-induced gelation of milk (Hindle & Wheelock, 1970). For both the SM-NaCas and SM-SMP milk samples, the level of 12% TCA-SN increased curvilinearly and plateaued to a final level of ~6.9 mg/100 g milk (data not shown) at ~27 min after rennet addition; this level of TCA-SN was comparable in magnitude to that reported previously for bovine milk (Hindle & Wheelock, 1970). There was no significant difference in the final levels of 12% TCA SN between the SM-SMP and SM-NaCas milk samples, suggesting no impact of ingredient on rennet-induced hydrolysis and that variation in rennet-induced proteolysis was not the reason for the inferior rennet gelation characteristics of SM-NaCas milk.

3.3.6. Heat coagulation time (HCT)

All milk samples exhibited a type A HCT/pH profile (Huppertz, 2016), with maximum stability in the pH region pH 6.6-6.7 and minimum at pH 6.8-7.0 (Figure 3.3). Milk protein fortification with NPC instead of SMP lowered the heat stability in the pH range 6.2 to 6.6, shifted the pH of maximum heat stability from 6.6 to 6.7, and increased the heat-stability in the region 6.7-7.2. The higher level of NPN and, hence, urea, which constitutes ~60% of NPN in milk (Phelan et al., 1982), in the SM-SMP milk may be a contributory factor to its relative high heat stability at pH 6.2-6.6 compared to SM-NPC milk (Shalabi & Fox, 1982). Muir and Sweetsur (1977) found that the addition of urea to milk at a level of 25 mg/100 g enhanced the heat stability significantly in the pH region 6.3-6.7. The estimated levels of urea in the SM-SMP and SM-NPC milks are 138 and 105 mg/100 g, respectively. The higher heat stability of the SM-NPC compared to the SM-SMP at 6.7-7.2 may be associated with its lower level of lactose and whey protein-to-casein ratio (Huppertz, 2016;

Singh & Fox, 1987). The addition of β-lactoglobulin to serum protein-free casein micelle dispersions was found to convert the HCT/pH curve from a type B to type A (Singh & Fox, 1987), whereby the heat stability is enhanced in the acid pH region (6.2-6.7) and reduced in the region of 6.8-7.2.

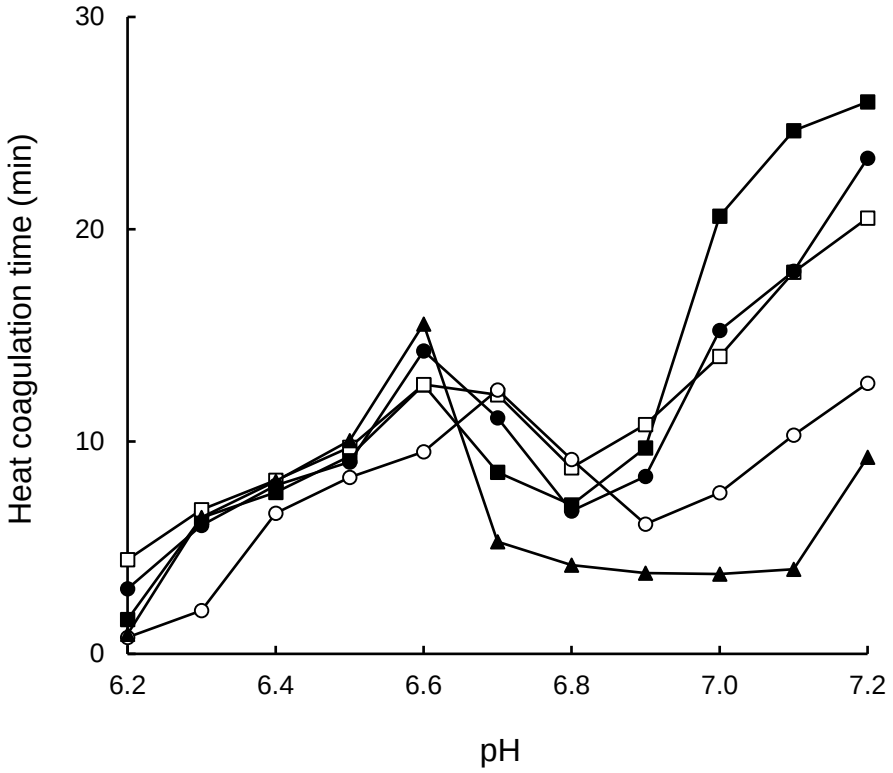

Figure 3.3. Heat coagulation time of milk samples in which protein content was fortified to 3.3 to 4.1% protein with different ingredients: skim milk powder, SMP (▲); native phosphocasein, NPC (○); calcium caseinate, CaCas (□);calcium-reduced phosphocasein, CaRPC (●); or sodium caseinate, NaCas (■).

The HCT of the SM-NaCas, SM-CaCas and SM-CaRPC milk samples was generally similar to the SM-SMP milk at pH 6.2-6.6 but was significantly higher than that of SM-SMP at pH 6.7-7.2 or than the SM-NPC at pH 6.9-7.2. Analogously, Cho and Singh (1999) observed an increase in the heat stability of recombined milk, formulated by blending an aqueous milk fat emulsion and reconstituted SMP, over the pH range 6.4 to 7.1, when the emulsion was stabilized using NaCas instead of

SMP or whey protein concentrate. This is perhaps surprising on consideration that the minimum in the heat stability curve of bovine milk (pH 6.8-7.0) is generally considered to be due to the dissociation of κ-casein from the micelle to the serum where it forms soluble complexes with β-lactoglobulin and the increased susceptibility of the remaining κ-casein-depleted micelles; hence, it might be expected that the heat stability of the SM-NaCas and SM-CaRPC in that context would be lower than the SM-SMP or SM-NPC at pH values in the range 6.7-6.9. This anomaly probably reflects the interactive contributions of different factors on heat stability of milk. The high heat stability of SM-NaCas, SM-CaCas and SM-CaRPC compared to SM-SMP at the higher pH values is consistent with their low levels calcium and phosphorous (Kaushik et al., 2015; Mohammed & Fox, 1986), lower lactose level, and higher proportion of non-sedimentable casein (Table 3.3); the low [Ca^{2+}] in the SM-NaCas fortified milk is also likely to enhance stability (Sievanen et al., 2008). It is also possible that the relatively high heat stability of SM-NaCas and SM-CaRPC at pH values in the region of the minimum heat stability may be associated with the high level of soluble (non-sedimentable) casein which may physically impede, or partially block, the aggregation of κ-casein depleted micelles. However, a better understanding why fortified milk (e.g., SM-NaCas) with a relatively high level of soluble casein (~18% of total casein) had a higher heat stability at pH 6.7-7.2 than fortified milk (e.g., SM-SMP) with a significantly lower level of soluble casein (~6% of total casein) would necessitate further research, for example examination of the casein profile of the milk samples and the corresponding supernatants before and after heat-induced coagulation at 140°C as a function of pH prior to heating.

3.3.7. Ethanol stability

The ethanol stability of all samples increased with pH in the range 6.2 to 7.2 (Figure 3.4a). The type of ingredient used for protein fortification significantly affected the ethanol stability at pH 6.4 and 6.6. At pH 6.4, the stability of the SM-NaCas, SM-CaCas or SM-CaRPC milk samples was significantly higher than that of the SM-SMP or SM-NPC samples, while at pH 6.6, stability was highest for SM-NaCas and SM-CaCas, lowest for SM-SMP, and intermediate for SM-NPC and SM-CaRPC (Figure 3.4a). The enhancing effect of NaCas, CaCas and CaRPC on the ethanol stability at pH 6.4 is likely associated with the reduction in both the proportion of sedimentable casein and the ratio of calcium-to-sedimentable casein; linear regression analysis showed a significant inverse relationship between stability at 6.4 and sedimentable casein ($r = 0.99$; $df = 13$). The significantly lower level of $[Ca^{2+}]$ in SM-NaCas milk compared to SM-SMP milk may also contribute to its higher ethanol stability at pH 6.4. Analogously, increasing the concentration of ionic calcium has been found to reduce ethanol stability of milk in the pH region 6.4-6.9 (Davies & White, 1958; Horne & Muir, 1990; Horne & Parker, 1981). A higher ratio of micellar (sedimentable)-to-soluble calcium is expected to enhance casein aggregation (Carr, Munro, & Campanella, 2002; Sood et al., 1979). Conversely, depleting the level of micellar calcium phosphate, by dialysis of milk samples against phosphate-free simulated milk ultrafiltrate, or by the addition of the calcium chelating salt ethylenediaminetetraacetic acid (EDTA), has been found to enhance ethanol stability in the pH range 6.4 to 6.9 (Horne & Parker, 1981; Mohammed & Fox, 1986).

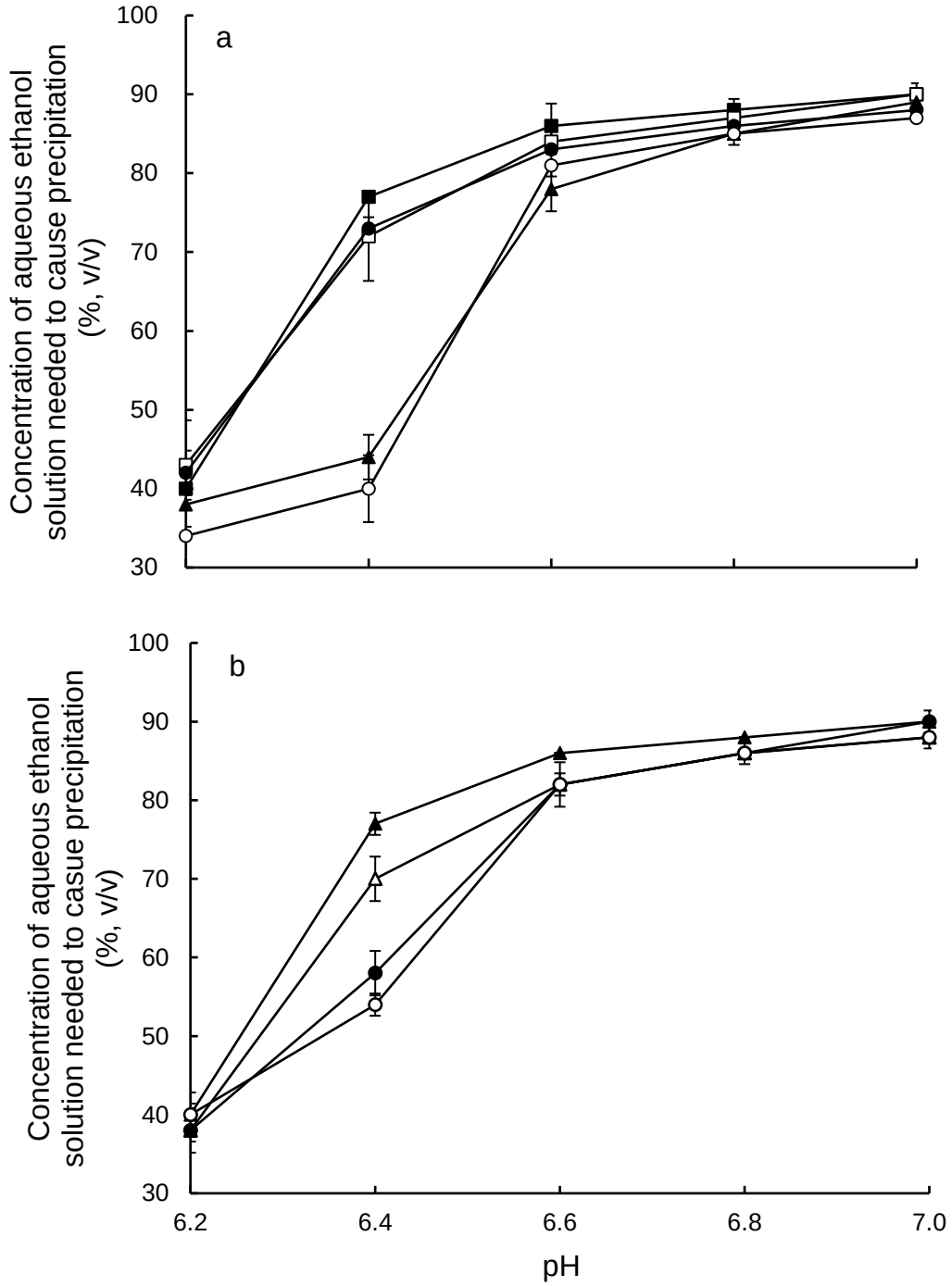

Figure 3.4. Ethanol stability of (a) skim milk in which protein content was fortified from 3.3 to 4.1% protein using different ingredients: skim milk powder, SMP (▲), native phosphocasein, NPC (○), calcium caseinate, CaCas (□), calcium-reduced phosphocasein, CaRPC (●) or sodium caseinate, NaCas (■); and (B) sodium caseinate-fortified milk in which the lactose content was increased by addition of lactose powder: 4.5% (▲), 4.9% (△), 5.3% (●) or 5.8% (○).

Another major difference in gross composition between the SM-SMP and SM-NaCas samples, which had the lowest and highest ethanol stabilities at pH 6.4 respectively, was lactose content, which was significantly higher in the former (~5.8%) than in the latter (~4.5%). To determine the potential contribution of the difference in lactose concentration to ethanol stability, the lactose content of the SM-NaCas milk was increased incrementally from 4.5 to 5.8% by adding lactose powder while retaining the protein level constant at 4.1%, through the replacement of water with lactose powder during the preparation of skim milk base portion of the fortified milk. The ethanol stability at pH 6.4 decreased significantly with lactose content (Figure 3.4b), suggesting that the difference in ethanol stability between SM-SMP and SM-NaCas milk samples was at least partly due to the difference in lactose content. Another factor contributing to the lower ethanol stability of the SM-SMP milk at pH 6.4 and 6.6, compared to the SM-NaCas, SM-CaCas or SM-CaRPC milk samples, may be its higher level of NPN. Davies and White (1958) reported that the ethanol stability of early lactation milk decreased as the level of NPN increased.

3.4. Conclusion

The use of different milk-protein powders to for milk protein fortification had a notable effect on heat stability, ethanol stability and rennet-induced gelation of the fortified milk samples. These differences were associated with corresponding changes in the proportions of sedimentable and non-sedimentable Ca, P and casein. The results indicate the importance of the degree of casein mineralisation and ratio of calcium-to-phosphorus of ingredients used for fortification of skim milk or reconstituted skim milk in various applications. Hence, a low degree of mineralisation and Ca-to-P ratio would be conducive to the formation of more heat-

and ethanol-stable beverages, but would be detrimental to rennet-induced gelation of recombined milk.

3.5. Acknowledgements

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Chapter 4

Addition of sodium caseinate to skim milk increases non-sedimentable casein and causes significant changes in rennet-induced gelation, heat stability and ethanol stability

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Abstract

The protein content of skim milk was increased from 3.3 to 4.1% (w/w) by the addition of a blend of skim milk powder (SMP) and sodium caseinate (NaCas), in which the weight ratio of SMP to NaCas was varied from 0.8:0.0 to 0.0:0.8. Addition of NaCas increased the levels of non-sedimentable casein (from ~6 to 18% of total casein) and calcium (from ~36 to 43% of total calcium) and reduced the turbidity of the fortified milk, to a degree depending on level of NaCas added. Rennet gelation was adversely affected by the addition of NaCas at 0.2% (w/w and completely inhibited at NaCas ≥0.4% (w/w). Rennet-induced hydrolysis was not affected by added NaCas. The proportion of total casein that was non-sedimentable on centrifugation (3,000 *g* for 1 h at 25°C) of the rennet-treated milk after incubation for 1 h at 31°C increased significantly on addition of NaCas at ≥0.4% (w/w). Heat stability in the pH range 6.7 to 7.2 and ethanol stability at pH 6.4 were enhanced by the addition of NaCas. It is suggested that the negative effect of NaCas on rennet gelation is due to the increase in non-sedimentable casein which upon hydrolysis by chymosin forms into small non-sedimentable particles which physically come between, and impede the aggregation of, rennet-altered *para*-casein micelles, and thereby inhibit the development of a gel network.

Key words: milk, protein fortification, dairy ingredients, processing characteristics

4.1. Introduction

Milk protein powders are extensively used as ingredients because of their techno-functional and nutritional properties. Applications include their use as ingredients in high protein beverages, nutritional beverages (e.g., for children), formulated and consumer foods, and recombined milks for the preparation of cheeses and fermented milk products (Gilles & Lawrence, 1981; Lagrange et al., 2015; McSweeney et al., 2013). The techno-functionalities required vary considerably according to application and may include water binding capacity, emulsification, heat-stability, ability to undergo gelation (e.g., on heating, acidification or rennet-treatment) and structure formation. In many of these applications, milk proteins are exposed to various unit operations (including acidification, heating, rennet-treatment, dehydration) and environments (e.g., food matrices differencing in solvent quality) that challenge their stability and functionality (Agarwal et al., 2015). The different functional requirements during food processing and formulation are met through the supply of a range of ingredients differing in protein type and content, extent of protein denaturation, degree of mineralization, and composition.

Skim milk powder (SMP) and sodium caseinate (NaCas) are widely used ingredients. They differ in method of manufacture, protein structure and degree of mineralisation. The manufacture of NaCas involves pH adjustment of the milk to the isoelectric point, precipitation of the casein and whey separation, washing and concentration, addition of sodium hydroxide to readjust the pH of the casein from ~6.8 to 7.0, and drying (Carr & Golding, 2016). During acidification, essentially all of the colloidal calcium phosphate,

which contributes to the self-assembly of the casein into micelles, is dissolved resulting in the dissociation of the casein micelles into smaller particles referred to as sub-micelles. Analysis of NaCas indicates significantly lower ratios of calcium- and phosphorous-to-casein compared to native casein in milk and the occurrence of the casein in the form of particles (~10 nm compared to ~150-200 nm in the native casein micelle) (O'Connell & Fox, 2000). In contrast, the structure of the casein and its degree of mineralization in SMP is not affected by the method of manufacture, which involves evaporation and drying of the milk.

These differences in casein structure and degree of mineralization are likely to impact rennet gelation, a critical parameter in the manufacture of cheese. Gaygadzhiev et al. (2012) found that the rennet-induced gelation of milk was impaired by the addition of 0.05% (w/w) NaCas and completely inhibited at a level ≥0.2% (w/w). The authors suggested the inhibitory effect of NaCas was likely due to the adsorption of the rennet-hydrolysed NaCas to the surface of the *para*-casein micelle and the concomitant increase in steric and electrostatic repulsion which impeded aggregation of the latter. Subsequently, Nair and Corredig (2015) found that the addition of 0.6% (w/w) NaCas to milk concentrated 3-fold had no effect on rennet gelation when the milk had been concentrated by ultrafiltration, but severely impeded gelation when the milk had been concentrated quiescently by osmotic concentration using polyethylene glycol. The dependence on the method of concentration was attributed to potential differences in the extent of rearrangement of the native micelles during concentration which affected their interaction with the added NaCas and the degrees to which it became adsorbed at the surface of, or

incorporated into, the micelle. Thomar and Nicolai (2015) reported that the addition of NaCas to an aqueous dispersion of native phosphocasein powder (NPC, 1.5% protein, w/w) promoted dissociation of casein, calcium and phosphorous from the micelle to a degree that increased with weight fraction of added NaCas.

The heat stability of dairy proteins is important in products such as ultra-high temperature (UHT) processed milk, infant milk formula and coffee whiteners. Consequently, heat stability of milk and the factors affecting it have been extensively studied (Huppertz, 2016). Comparatively little information is available on the effect of adding NaCas to milk on the heat stability. Cho and Singh (1999) observed an increase in the heat stability (140°C) of recombined milk, formulated by blending an aqueous milk fat emulsion and reconstituted SMP, over the pH range 6.4 to 7.1, when the emulsion was stabilised using NaCas instead of SMP or whey protein concentrate.

Cream liqueurs are formulated mainly from cream (33-40%, w/w), ethanol (~12-15% v/v), sucrose (~18.5%, w/w), milk protein (typically ~3.5%, w/w, NaCas) and water (~25-30%, w/w) (Muir, 1988). The ethanol stability of NaCas is of particular relevance in emulsion stabilization and control of storage-related flocculation, thickening or gelation. O'Kennedy et al. (2001) reported that the ethanol stability of a 3% (w/w) aqueous dispersion of NaCas depended on pH and ionic strength.

The principal objective of the current study was to investigate the effect of incrementally increasing NaCas from 0 to 0.8% (w/w) on the rennet

gelation, heat stability and ethanol stability of fortified milk (4.1% protein, w/w) prepared by adding a blend of SMP and NaCas to skim milk (3.3% protein, w/w) in which the weight ratio of protein from SMP-to-NaCas was varied from 0.8:0.0 to 0.0:0.8. A secondary objective was to relate the effects of NaCas on the above properties to changes in the partition of protein and minerals between the sedimentable and non-sedimentable phases obtained on ultracentrifugation of the fortified milk at 100,000 g.

4.2. Materials and methods

4.2.1. Milk protein ingredients

Milk protein ingredients used included extra low-heat skim milk powder (<4% of total whey protein denatured) and NaCas. The respective levels of total protein, casein, whey protein, lactose, calcium and phosphorous contents of the SMP and NaCas are shown in Table 4.1.

Table 4.1. Composition of dairy ingredients used for fortifying the protein content of skim milk[1]

	Ingredients	
Composition[2]	SMP	NaCas
Total protein (%, w/w)	36.4	87.9
Casein (% TP)	82.8	97.0
Whey protein (% TP)	11.2	1.2
Lactose (%, w/w)	46.35	1.27
Ca (mg /100 g)	1295	193
P (mg /100 g)	913	687

[1]Ingredient code: SMP, low heat skim milk powder; NaCas, sodium caseinate.
[2]TP, total protein; Ca, calcium; P, phosphorus.

4.2.2. Preparation of milk

A skim milk base (3.3%, w/w, protein) was prepared from reconstituted SMP, rather than using fresh skim milk, to ensure a compositionally consistent starting material and to avoid the potential confounding effects of seasonal changes in milk quality and composition on the measured characteristics, during replicate trials. The skim milk base was prepared by dispersing the SMP in distilled water at 50°C while shearing at 6,300 rpm for 5 min using a high-shear mixer (Silverson model L4RT, Silverson, Chesham, UK) until the powder was visually dispersed, as described in Chapter 3. The sample was then placed at 4°C for 22 h to allow for hydration of the protein. The preparation of the fortified milk (4.1%, w/w, protein) involved adding the blend of SMP and NaCas powders to the base skim milk in sufficient quantities to increase the protein content from 3.3 to 4.1% (w/w). The ratio of protein derived from the SMP to protein from the NaCas during fortification was varied as follows: 0.0:0.8, 0.2:0.6, 0.4:0.4, 0.5:0.3, 0.6:0.2, 0.7:0.1, and 0.8:0.0. Following addition of SMP or NaCas, the temperature was maintained at 50°C while shearing for a further 20 min. Sodium azide was added at a level of 0.02% to all milk samples prior to cooling for preservation purposes.

The cold ageing effect of holding milk samples at 4°C was reversed by incubating the milk at 40°C for 30 min prior to all assays (Fox, 1969).

4.2.3. Preparation of milk ultracentrifugate

Milk ultracentrifugate was prepared by centrifugation of milk at 100,000 *g* for 1 h at 25°C, and decantation of the supernatant through glass wool.

4.2.4. Composition of milk and milk ultracentrifugate

The fortified milk (4.1% protein, w/w,) was analysed for fat and total solids using CEM SMART Trac II (CEM, North Carolina, USA), total protein by Kjeldahl (International Dairy Federation, IDF, 2001a) and lactose by the FOSS MilkoScan FT+ (Foss Electric A/S, Hillerød, Denmark). Calcium (Ca) content in milk and ultracentrifugate was measured by atomic absorption spectrometry (Chapter 2); phosphorous (P) was assayed by molecular absorption spectrometry (Chapter 2). Total nitrogen (N), non-casein nitrogen and the non-protein nitrogen (NPN) in milk and ultracentrifugate were determined using standard IDF methods (IDF, 2001a, b). Concentration of ionic calcium ($[Ca^{2+}]$) was measured using sensION+ 9660C Calcium Combination Ion Selective Electrode (Hach Lange, Barcelona, Spain). Potassium chloride (3M) was added to the milk at a level of 1% (v/v) and the milk was assayed for Ca^{2+} concentration while stirring (Chapter 3).

Protein profile was determined by reversed-phase high pressure liquid chromatography (RP-HPLC; Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA) using a 300 SB-C18 RP Poroshell column (Agilent Technologies, Santa Clara, CA, USA), according to the method of Visser et al. (1991).

4.2.5. Turbidity (τ)

Fortified milk was diluted 1:10 using milk permeate prepared by lab scale ultrafiltration of skim milk (NovaSet-LS cassette, 10kDa, 0.1 m^2, ProStream modified polyethersulfone membrane, TangenX Technology Corporation, MA, USA). The diluted sample was agitated gently and the

turbidity was measured at 860 nm using 2100N Turbidimeter (Hach Lange GmbH, Willstätterstraße, Germany).

4.2.6. Casein micelle size

Fortified milk was diluted 1:10 in simulated milk ultrafiltrate, prepared according to Jenness and Koops (1962). The casein micelle size was then measured using the Malvern Zetasizer Nanoseries Nano-ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK) with a backscatter angle of 173° at 25°C. The mean average size (z-average) was measured using an intensity distribution.

4.2.7. Rennet gelation

Milk was adjusted to pH 6.55 and tempered to 31°C. Chymosin (single strength Chy-Max plus, 200 IMCU/mL; Chr. Hansen, Hørsholm, Denmark) was diluted 1 in 20 with distilled water, and added at a level of 421 μl/100 mL to the milk sample and mixed for 30 s; this level of rennet addition equates to 0.21 mL Chy-Max plus per L milk with 4.1% protein. The storage modulus (G′) was measured dynamically as a function of time over 1 h at a strain of 0.025 and a frequency of 1 Hz in a controlled stress rheometer (Carri-Med, type CSL^2_{500}, TA instruments, New Castle, DE, USA) (Guinee et al., 2006). The following parameters were calculated from the resultant G′/time curve: gelation time, defined as the time for G′ to reach a value of ≥0.2 Pa; maximum curd firming rate, the maximum slope of the curve; and gel firmness at 60 min after rennet addition (G'_{60}).

The level of proteolysis of κ-casein in the rennet-treated milk at pH 6.55 was determined by measuring the change in the level of total N soluble in 12% (w/v) TCA (trichloroacetic acid) at different time intervals during the 60-min incubation period

(Hindle and Wheelock, 1970). Following incubation for 60 min at 31°C, the rennet-treated milk was centrifuged at different *g* forces (3,000-100,000 *g*) for 1 h at 25°C to determine if casein which was non-sedimentable in the milk prior to rennet treatment sedimented following hydrolysis with chymosin. The resultant supernatant was filtered (Chapter 3). The resultant whey (serum) was analysed for protein, calcium and phosphorous, as described above for milk.

4.2.8. Heat stability and ethanol stability

Milk samples were adjusted to different pH values in the range from 6.2 to 7.2 (0.1 pH unit increments) at room temperature using 0.1 N HCl or NaOH. A sub-sample was placed in 4 mL heat-resistant tube which was capped with a rubber bung and placed and secured in a metal rack. The loaded rack was placed in the temperature-controlled oil bath at 140°C and rocked gently at a constant frequency (7 oscillations/ min) as described in Chapter 2. The analysis at each pH value was performed in duplicate and the mean values for each pH were used to construct a heat stability/pH curve.

The ethanol stability of a subsample of each milk, adjusted to pH values in the range 6.2 to 7.0, was measured as the concentration of ethanol required for visual flocculation on blending the milk with ethanol solutions of varying strength (30-98% ethanol, v/v) at a volume ratio of 1:4.8 (Horne and Muir, 1990).

4.2.9. Statistical analysis

Duplicate batches of each treatment milk, containing different levels of added sodium caseinate, were prepared on separate occasions. The data were analysed using a randomized complete block design incorporating the 7 different milk treatments (fortified milk with different proportions of SMP and NaCas) and 2 blocks (replicate

trials). The effect of treatment was determined by applying analysis of variance using the general linear model procedure of SAS 9.3 (SAS Institute, 2011). Tukey's multiple-comparison test was used as a guide for pair comparisons of the treatment means and the level of significance was determined at $P < 0.05$. The data were also analysed by linear regression to establish potential correlations between measured parameters.

4.3. Results

4.3.1. Composition of milk and milk ultracentrifugate

Increasing NaCas, as a proportion of added protein (i.e., reducing the ratio of SMP:NaCas), resulted in significantly lower levels of whey protein (as % total protein), lactose, Ca, [Ca^{2+}] and P (Table 4.2); moreover, when added NaCas was increased from 0 to ≥0.4% (w/w), casein (as a % of total protein) increased significantly. This trend is consistent with the lower levels of lactose, whey protein, Ca and P in NaCas compared to SMP.

Table 4.2. Compositional and physico-chemical characteristics of skim milk fortified to 4.1% protein (w/w) using a blend of sodium caseinate and skim milk powder[1,2,3]

	Skim milk	Fortified milk: level of added sodium caseinate (%, w/w)						
Item		0.0	0.1	0.2	0.3	0.4	0.6	0.8
Milk								
DM (%, w/w)	8.78	11.3^{a}	11.3^{a}	10.8^{ab}	10.7^{b}	10.4^{b}	9.6^{c}	9.5^{c}
TP (%, w/w)	3.31	4.1^{a}	4.1^{a}	4.1^{a}	4.1^{a}	4.1^{a}	4.1^{a}	4.1^{a}
Casein (% TP)	76	76.0^{b}	79.6^{ab}	80.3^{a}	79.8^{ab}	80.1^{a}	82.0^{a}	82.8^{a}
Whey protein (% TP)	18.31	18.3^{a}	15.5^{b}	15.2^{bc}	15.6^{b}	14.8^{bc}	13.9^{bc}	13.4^{c}
NPN (% TP)	5.66	5.7^{a}	4.9^{a}	4.8^{a}	4.6^{a}	4.7^{a}	4.1^{a}	3.8^{a}
Lactose (%, w/w)	4.47	5.8^{a}	5.6^{ab}	5.5^{ab}	5.4^{b}	5.1^{c}	4.8^{cd}	4.5^{d}
Ca (mg/100 g)	130	161^{a}	156^{a}	149^{b}	142^{c}	143^{bc}	136^{d}	132^{d}
Ionic Ca (mM)	ND	1.93^{a}	1.72^{b}	1.72^{b}	1.70^{b}	1.67^{bc}	1.67^{bc}	1.61^{c}
P (mg/100 g)	95	116^{ab}	118^{a}	108^{bc}	108^{bc}	105^{cd}	99^{d}	98^{d}
Particle size (nm)	166	170^{a}	172^{a}	174^{a}	172^{a}	171^{a}	171^{a}	172^{a}
Pellet obtained on ultracentrifugation								
Casein (% milk casein)	93.3	93.9^{a}	91.8^{ab}	90.8^{ab}	89.1^{abc}	85.3^{bc}	84.8^{c}	82.1^{c}
Ca (mg/g casein)	34.4	35.4^{a}	32.2^{ab}	30.2^{bc}	31.5^{b}	32.0^{ab}	28.8^{bc}	27.2^{c}
P (mg/g casein)	22.7	22.1^{a}	22.0^{a}	20.1^{abc}	20.2^{abc}	20.6^{ab}	17.3^{c}	17.8^{bc}

[1] The weight ratio of protein from skim milk powder to sodium caseinate in the powder blend used for fortification was varied from 0.8:0.0 to 0.0:0.8.
[2]Values within a row not sharing a common superscript (a-d) differ significantly ($P < 0.05$). Presented data are the means of duplicate batches for each milk.
[3]ND, not determined; DM, dry matter; TP, total protein; NPN, non-protein nitrogen; Ca, calcium; P, phosphorus.

Table 4.3. Composition of milk ultracentrifugate obtained on centrifugation of fortified milk (4.1% protein, w/w) with different level of added sodium caseinate[1,2,3,4]

	Skim milk	Fortified milk: level of added sodium caseinate (%, w/w)						
Item		0.0	0.1	0.2	0.3	0.4	0.6	0.8
Protein (%, w/w)	0.97	1.15^{bc}	1.14^{c}	1.17^{bc}	1.20^{bc}	1.26^{ab}	1.37^{a}	1.37^{a}
Casein (%, w/w)	0.17	0.19^{d}	0.27^{cd}	0.27^{cd}	0.38^{bc}	0.48^{abc}	0.51^{ab}	0.61^{a}
Casein (% milk casein)	6.7	6.1^{c}	8.2^{bc}	8.3^{bc}	11.7^{abc}	14.6^{ab}	15.2^{a}	17.9^{a}
Ca (mg/100 g)	49	58^{a}	59^{a}	59^{a}	51^{a}	53^{a}	53^{a}	57^{a}
Ca (% milk Ca)	37.7	35.8^{c}	38.1^{bc}	39.3^{ab}	36.2^{ab}	37.3^{bc}	39.3^{ab}	42.8^{a}
P (mg/100 g)	42	51^{a}	52^{a}	48^{a}	49^{a}	48^{a}	49^{a}	48^{a}
P (% milk P)	44.1	44.1^{b}	43.9^{b}	44.6^{a}	45.5^{a}	45.3^{a}	49.8^{a}	49.2^{a}

[1]The weight ratio of protein from skim milk powder to sodium caseinate in the powder blend used for fortification was varied from 0.8:0.0 to 0.0:0.8.
[2]Ultracentrifugate was obtained by centrifugation at 100,000 *g* at 25°C for 1 h.
[3]Values within a row not sharing a common superscript letter (a-d) differ significantly ($P < 0.05$). Presented data are the means of duplicate batches for each milk.
[4] Ca, calcium; P, phosphorus.

The concentration of casein in the ultracentrifugate (non-sedimentable casein) increased significantly with level of added NaCas (Table 4.3), from ~6.1% of total casein, or ~0.19 %, in fortified milk without NaCas to 17.9% total casein, or 0.61%, in fortified milk with 0.8% added NaCas. The level of casein in the ultracentrifugate of the fortified milk without added NaCas was similar to that of control (non-fortified) skim milk (Table 4.3). The proportions of individual caseins in the milk ultracentrifugate, expressed as % of the corresponding casein in milk, changed little on increasing added NaCas from 0.0 to 0.4% (w/w) but increased significantly on further increasing NaCas from 0.4 to 0.8% (Figure 4.1a). At 0.8% added NaCas, the proportions of individual caseins in the serum, expressed as a % of the corresponding level in milk, were in the following order: κ-casein (39%) > β-casein (28%) > α_{s1}-casein (17%) > α_{s2}-casein (16%). Expressed as % of total casein in the ultracentrifugate, κ- and α_{s1}- caseins changed with level of added NaCas, with the former decreasing and the latter increasing, respectively, at NaCas ≥0.3 % (w/w) ($P <$

0.05). The proportions of, κ-, β-, α_{s1}- and α_{s2}-caseins accounted for 29, 40, 24 and 7%, respectively, at 0.8% (w/w) NaCas (Figure 4. 1b).

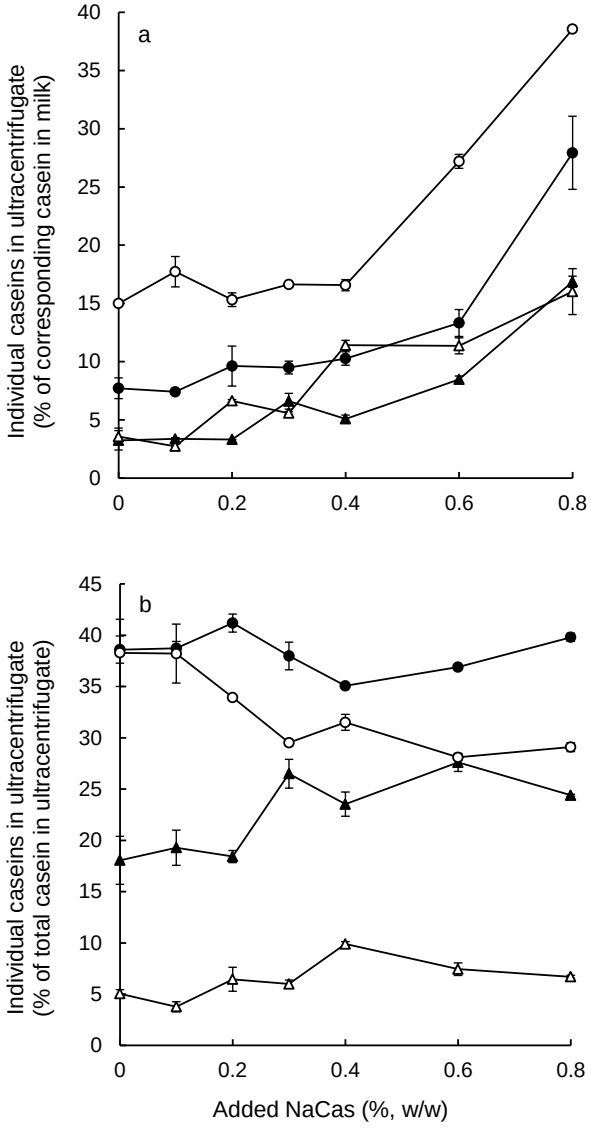

Figure 4.1. Effect of level of added sodium caseinate, NaCas, in fortified milk (4.1% protein, w/w) on the concentration of individual caseins in milk ultracentrifugate, expressed as % of the corresponding casein in milk (a) or as % of total casein in ultracentrifugate (b): α_{s1}- (▲), α_{s2}- (△), β- (●) and κ- (○) casein. Presented data are the means of duplicate batches of each treatment milk; error bars show standard deviation of the mean.

4.3.2. Particle size and turbidity (τ)

All milk samples showed a mono-modal particle size distribution and z-average of ~170 nm (Table 4.2), which is within the range reported by others, i.e., 130-200 nm (Holt & Muir, 1978; O'Connell & Fox, 2000; Glantz et al., 2010).

Turbidity of the fortified milk, diluted (1:10) in ultrafiltered milk permeate at 860 nm, decreased linearly from 1,537 NTU (nephelometric turbidity unit) to 1,360 NTU with increasing fraction of NaCas from 0 to 0.8% (w/w) (Figure 4.2).

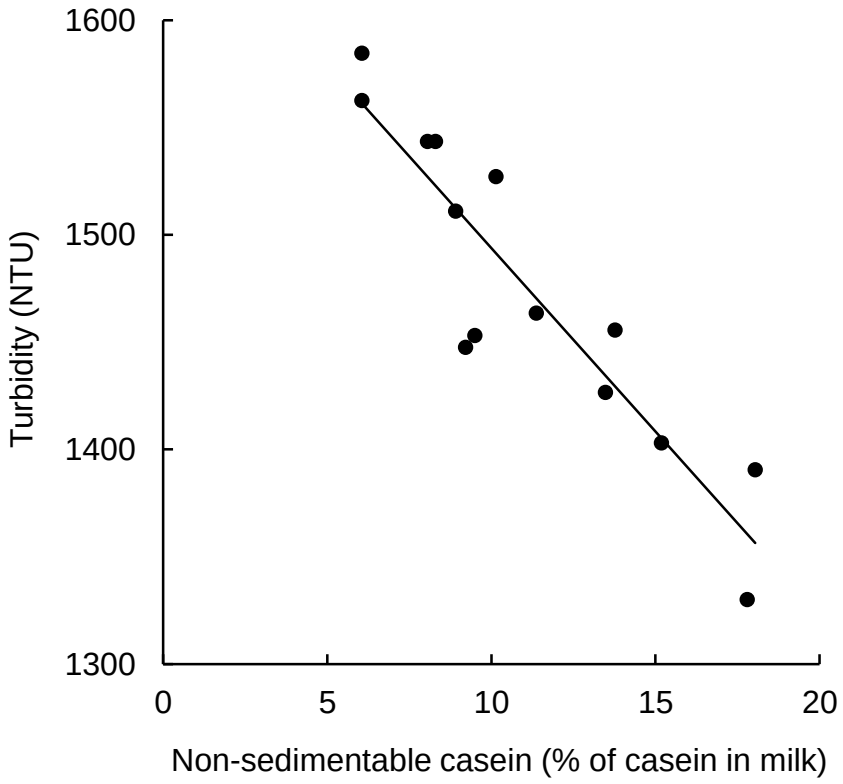

Figure 4.2. Relationship between turbidity and non-sedimentable casein in frotified milk containing 0 to 0.8% (w/w) added sodium caseinate, NaCas (see Table 4.2 for details). Presented data show values of duplicate batches of seven fortified milk samples with added NaCas ranging from 0 to 0.8%. NTU, ephelometric Turbidity Unit.

4.3.3. Rennet gelation properties

The addition of NaCas adversely affected the rennet gelation properties of the fortified milk, as reflected by a significant increase in the gelation time and reductions in G'_{60} and max curd firming rate (Figure 4.3). The value of G'_{60} decreased linearly (P <0.05) as the level of added NaCas was increased from 0.0 to 0.3% (w/w) and the milk did not gel when NaCas was added at levels ≥0.4% (w/w) (Figure 4.3).

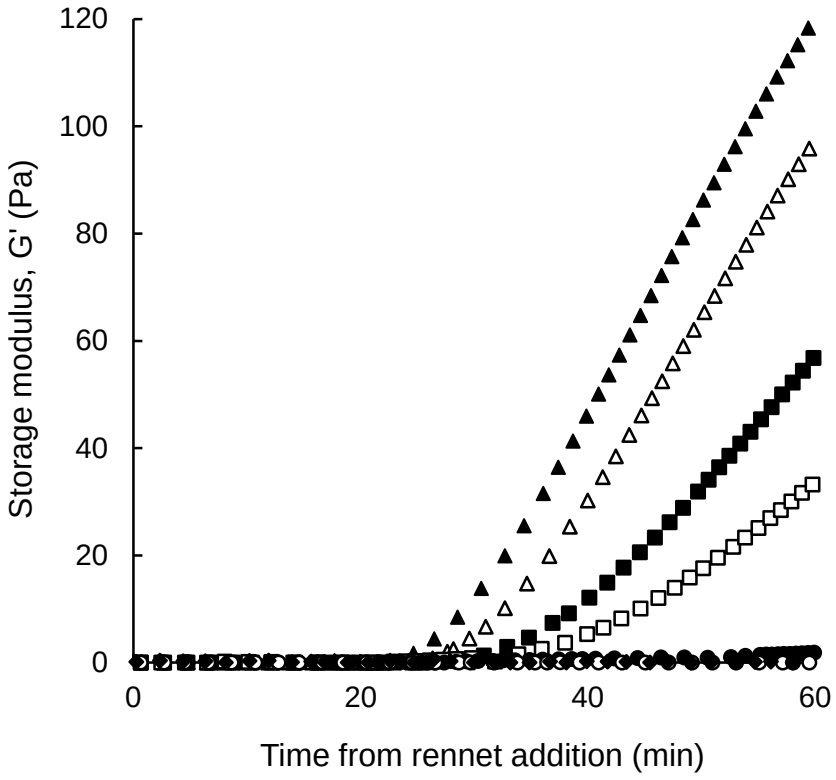

Figure 4.3. Development of storage modulus, G', in rennet-treated fortified milk (4.1% protein, w/w) containing different levels of added sodium caseinate, NaCas: 0 (▲), 0.1 (△), 0.2 (■), 0.3 (□), 0.4 (●), 0.6 (○) or 0.8 (◆).

4.3.4. Proteolysis during rennet gelation

The level of 12% (w/v) TCA-soluble N (SN) has been used as index of the degree of hydrolysis of κ-casein by the coagulant, and the formation of the resultant caseino-macropeptide, during rennet-induced gelation of milk (Hindle & Wheelock, 1970).

Rennet-treated milk with different levels of added NaCas was examined for the rate of formation of 12% (w/v) TCA-SN during incubation at 31°C to establish if the adverse effect of added NaCas at ≥0.3% (w/w) on rennet-induced gelation was due to inhibition of the primary-stage enzymatic hydrolysis of κ-casein, inhibition of the secondary-stage aggregation of the chymosin-altered micelles into a gel, or a combination of both. 12% (w/v) TCA-SN increased with incubation to 40 min, after which levels plateaued. The mean increase in 12% TCA-SN was ~6.9 mg/100 g milk after 40 min and was not significantly affected by level of added NaCas (Figure 4.4).

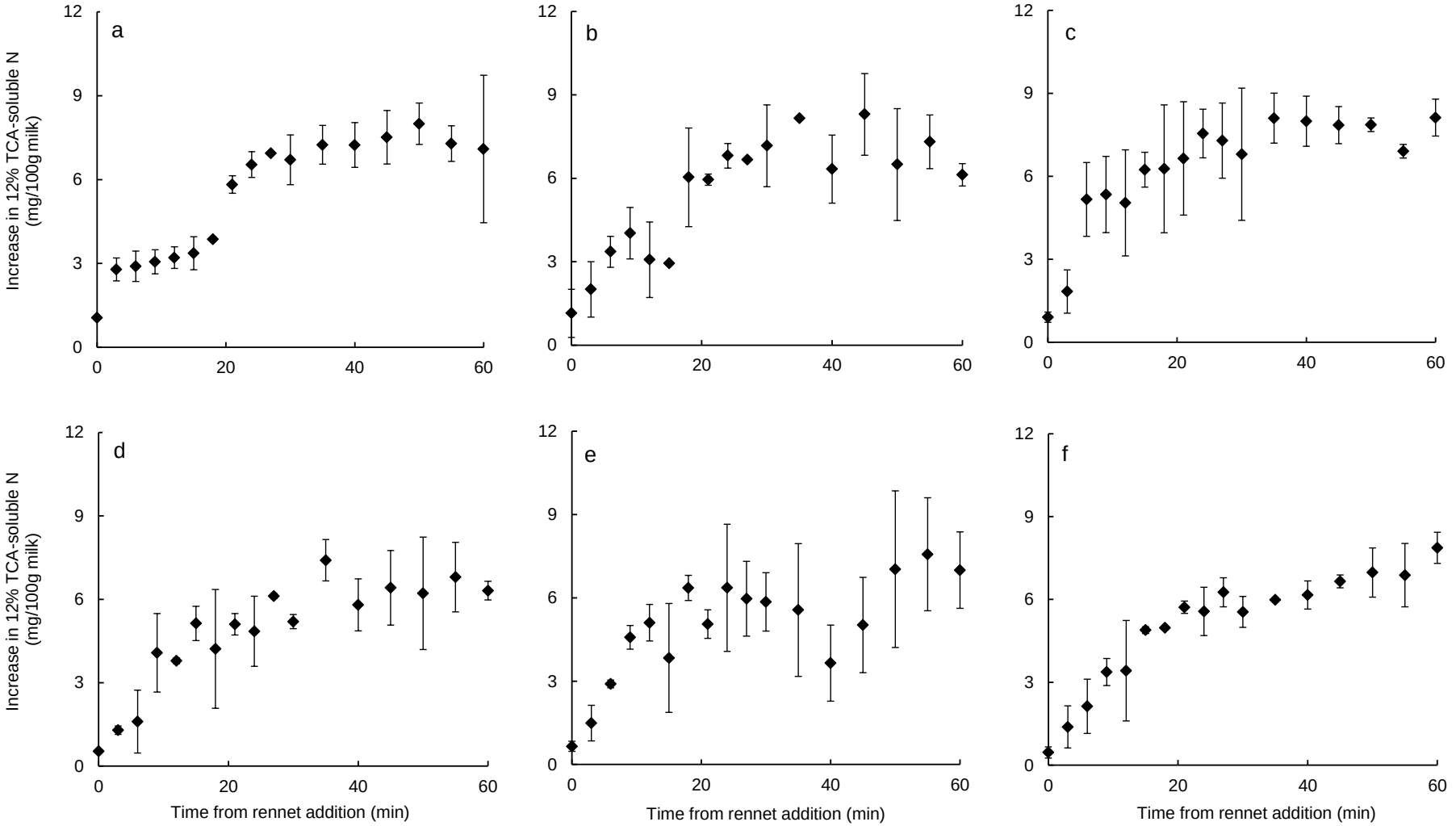

Figure 4.4. Increase in level of 12% (w/v) trichloroacetic acid (TCA)-soluble N (mg 100/g milk) in rennet-treated fortified milk (4.1% protein, w/w) containing different levels (%, w/w) of added sodium caseinate, NaCas: 0 (a), 0.1 (b), 0.2 (c), 0.4 (d), 0.6 (e) or 0.8 (f). Presented data are the means of duplicate batches of each fortified milk; error bars show standard deviation of the mean.

4.3.5. Appearance and composition of whey (serum) from rennet-treated milk samples

The whey phase obtained on centrifugation (3,000 *g*) of the rennet-treated milk obtained at 1 h after rennet addition (when the fortified milk without NaCas had formed a very strong gel, ~120 Pa) became progressively milky when the level of added NaCas was increased to ≥0.4% (w/w); essentially, the rennet-treated milk was similar in appearance and consistency (liquid) to the milk without added rennet. Simultaneously, soluble casein, Ca and P (as % of total casein, Ca and P, respectively) increased significantly (Figure 4.5a). Hence, despite similar levels of chymosin-induced proteolysis of the casein at all levels of NaCas addition, the casein remained largely soluble at 3,000 *g* when the level of added NaCas was ≥0.4% (w/w). However, the level of soluble casein decreased sharply ($P < 0.05$) with centrifugation force in the range 3,000 to 100,000 *g*, but still remained high in the milk with 0.8% (w/w) NaCas at ≤30,000 *g* (Figure 4.5b).

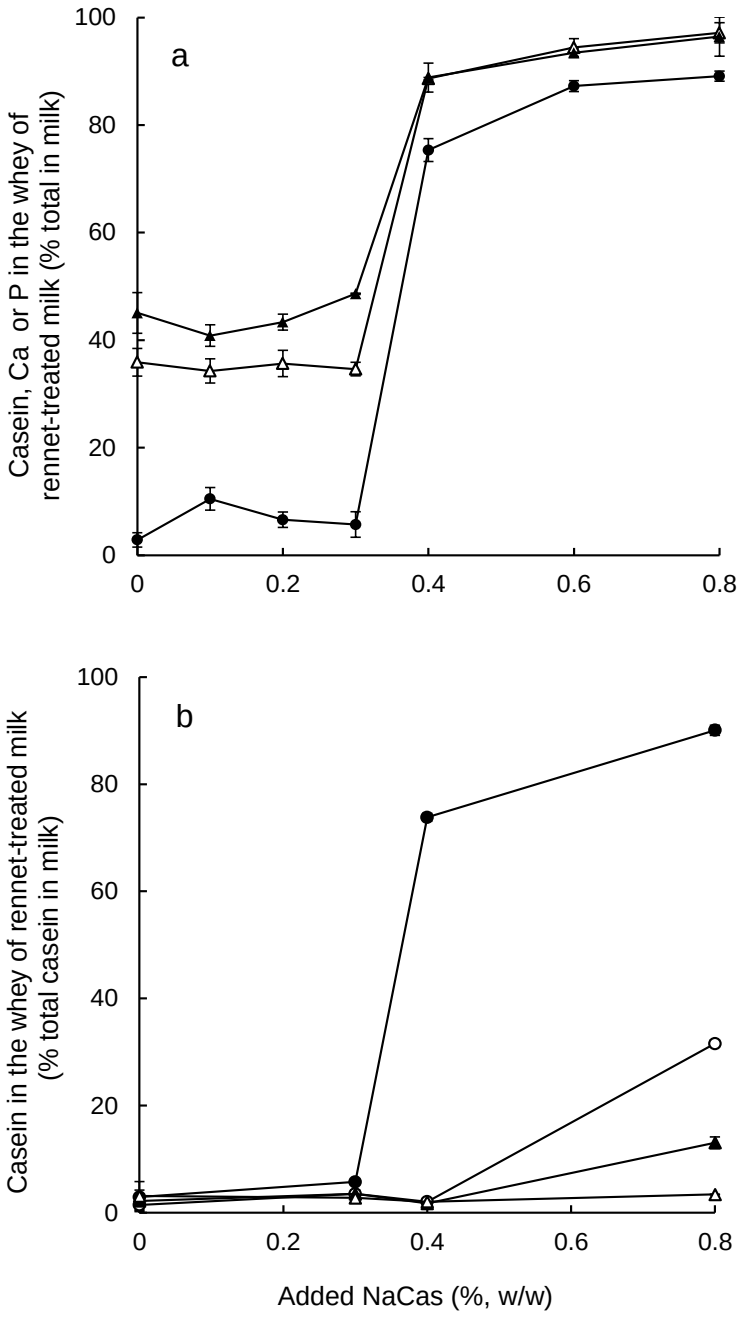

Figure 4.5. Effect of varying the level (%, w/w) of added sodium caseinate, NaCas, in fortified milk (4.1% protein) on the composition of whey obatined on centrifugation of the rennet-treated milk: (a) concentrations of casein (●), calcium (Ca, △) or phosphorus (P, ▲) in the whey (serum) obtained on centrifugation at 3,000 *g*; and (b) concentration of casein in the whey obtained on centrifugation at 3,000 (●), 12,500 (○), 30,000 (▲) or 100,000 (△) *g*. Presented data are the means of duplicate batches of each fortified milk; error bars show standard deviation of the mean.

4.3.6. Heat stability and ethanol stability

Increasing the level of NaCas added to the milk did not affect the heat stability profile, which for all milk treatments was type A with a maximum heat coagulation time at pH 6.6 and a minimum at pH 6.8 to 7.0. However, increasing added NaCas to ≥0.2% (w/w) coincided with a significant reduction in the maximum stability (pH 6.6), an increase in the heat stability at pH 6.7 to 7.2, a narrowing of the minimum stability pH zone, and a shift in the pH of minimum heat stability to lower pH (Figure 4.6a). The increase in heat stability was most pronounced at pH 7.0 and 7.1, and increased progressively with level of added NaCas.

The ethanol stability of all milk treatments increased with pH from 6.2 to 7.0. Ethanol stability at pH 6.4, 6.6 and 6.8 increased significantly with level of added NaCas. This effect was most pronounced at pH 6.4 (Figure 4.7a) where ethanol stability was positively correlated with non-sedimentable casein (as a proportion of total casein) (Figure 4.7b).

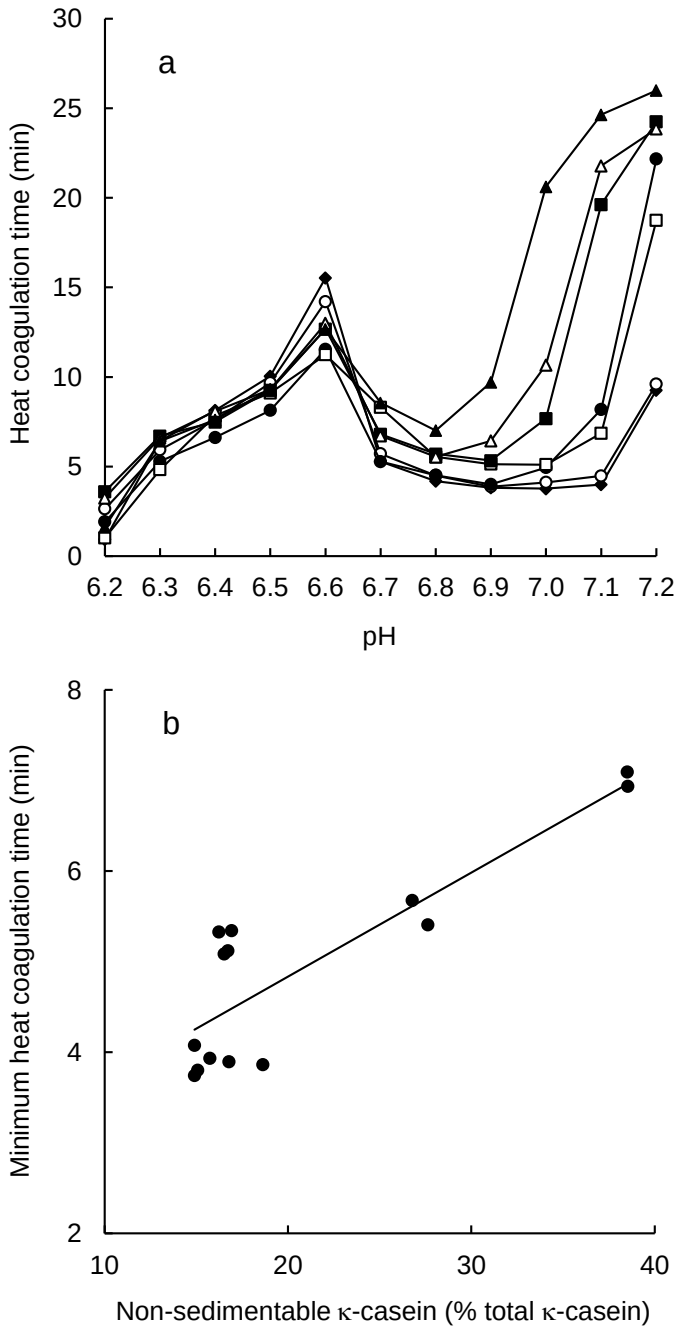

Figure 4.6. (a) Heat stability of fortified milk (4.1% protein, w/w) containing different levels (%, w/w) of sodium caseinate, NaCas: 0 (◆), 0.1 (○) 0.2 (●), 0.3 (□), 0.4 (■), 0.6 (△), or 0.8 (▲); presented data show the means of duplicate batches of each fortified milk. (b) Minimum heat coagulation time of fortified milk samples (at pH >6.6) as a function of non-sediemntable κ-casein content (% total κ-casein in milk). Presented data show values of duplicate batches of seven fortified milk samples with added NaCas levels ranging from 0 to 0.8%.

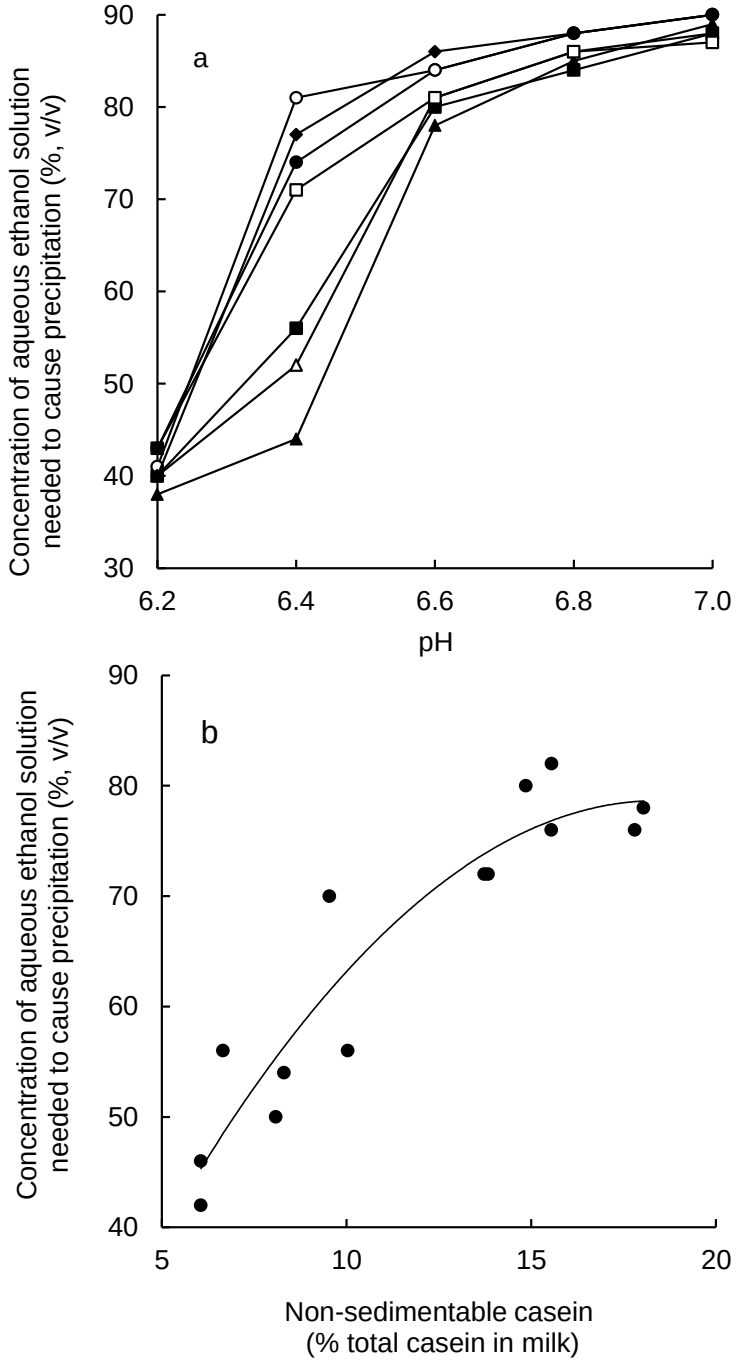

Figure 4.7. (a) Concentration of ethanol required to induce flocculation of fortified milk samples (4.1% protein, w/w) containing different levels (%, w/w) of added sodium caseinate, NaCas: 0 (▲), 0.1 (△), 0.2 (■), 0.3 (□), 0.4 (●), 0.6 (○) or 0.8 (◆); presented values are the means of duplicate batches of each fortified milk. (b) Ethanol stability of the fortified milk samples at pH 6.4 as a function of non-sediemntable casein content (% total casein in milk); presented data show values of duplicate batches of seven fortified milk samples with added NaCas ranging from 0 to 0.8% (w/w).

4.4. Discussion

The current study focussed on the compositional and stability characteristics of fortified milk (4.1% protein, w/w) prepared by increasing the protein content of skim milk from 3.3 to 4.1% using a blend of SMP and NaCas, in which the weight ratio of protein from SMP-to-NaCas was varied from 0.0:0.8 to 0.8:0.0. Increasing added NaCas led to a significant increase in non-sedimentable casein, from ~0.19 to 0.61% (w/w), and a change in the proportion of individual caseins comprising the non-sedimentable casein. κ-Casein and α_{s1}-casein, as proportions of the non-sedimentable casein, decreased ($P < 0.05$) and increased ($P < 0.05$), respectively as the level of added NaCas was increased from 0.4 to 0.8% (w/w). Added NaCas also increased the proportions of non-sedimentable Ca and P ($P < 0.05$). Nevertheless, the mean particle size (z-average) and the area of the size intensity peak in the protein-fortified milk did not change with level of NaCas. Likewise, Gaygadzhiev et al. (2012) found that the addition of 0.1% (w/w) NaCas to skim milk did not affect the apparent diameter of the casein micelle, as measured using dynamic light scattering. In contrast, the turbidity decreased significantly as the level of added NaCas was increased, reflecting a reduction in the intensity of light scattering by casein particles; this trend concurs with the reduction in sedimentable casein as a proportion of total casein. Similarly, Thomar and Nicolai (2015) reported an increase in the non-sedimentable casein content of an aqueous dispersion of NPC (1.5% protein, w/w) on addition of NaCas; the turbidity (685 nm) of the dispersion decreased proportionally with weight fraction of NaCas added. The authors postulated that added NaCas behaved essentially as a calcium-chelating salt which caused dissociation of the casein micelles in the NPC. Earlier, Parker et al. (2005) found that the level of non-sedimentable casein in reconstituted skim milk, to which NaCas was

added, increased as the level of added NaCas was increased from 0.0 to 1.0% (w/w), but decreased on heating the milk to 80°C for 30 min. The authors concluded that NaCas added to skim milk partially remained non-sedimentable in the form of soluble complexes (not as sedimentable casein micelle-like particles) which on heating to 80°C become associated with the denatured whey protein, probably by binding with the κ-casein of NaCas and preventing some of the κ-casein on casein micelle (originally present in milk) from dissociation. Parker et al. (2005) also proposed that the hydrophobic α_s- and β-casein from NaCas may interact with casein micelles below hydrodynamic surface of casein micelle, if the interior of the casein micelles is accessible via the irregularities of the casein micelle surface. The current results showed that the increase in non-sedimentable casein on addition of NaCas to skim milk was less than the value expected, had all the added NaCas become non-sedimentable, for example an increase of 0.42% (w/w) on addition of 0.8% (w/w) NaCas. The results suggest that some of the NaCas added to the skim milk (0.8%, w/w) may have associated with the native casein micelles without altering their mean size (z-average), while the remainder, which increases with level of NaCas added, remained non-sedimentable in the ultracentrifugate. It is also possible that some of added NaCas forms into sub-micellar-type particles (e.g., <10 nm diameter) by self-assembly of molecules of NaCas in the presence of serum calcium. Pitkowski et al. (2009) reported casein which remained non-sedimentable on centrifugation (56,000 *g* for 90 min at room temperature) of aqueous solutions of NaCas, with different levels of added $CaCl_2$, was organized into small clusters (aggregates) with a radius of ~12 nm containing ~15 casein molecules.

The addition of NaCas at ≥0.4% (w/w) to skim milk resulted in failure of the milk to undergo rennet-induced gelation. Gaygadzhiev et al. (2012) made a similar

observation and hypothesised that the addition of NaCas to skim milk impaired rennet gelation by adsorbing at the interface of the casein micelle and enhancing steric and electrostatic repulsion between *para*-casein micelles. Our results showed that the level of 12% (w/v) TCA-SN in fortified milks with different levels of added NaCas (0 to 0.8%, w/w) was similar and, therefore, suggest that the rate and extent of hydrolysis of the caseino-macropeptide from the κ-casein was not a causative factor for the deterioration in rennet gelation as the level of added NaCas increased. This adverse effect of NaCas at ≥0.4% (w/w) on rennet-induced gelation may be partly associated with the reduction in [Ca^{2+}], and additionally with the decrease in ratio of sedimentable Ca-to-casein at higher levels (0.6 to 0.8%, w/w) of added NaCas (Singh et al., 1988). However, the increase in non-sedimentable casein in the milk is also likely to be a contributory factor to the negative impact of NaCas on rennet gelation, as confirmed by the milky appearance and the high proportion of total casein (~90%) and calcium (98%) that remained 'soluble' on centrifugation of the rennet-treated milk with ≥0.4% (w/w) NaCas at 3,000 *g*. The current results obtained on centrifugation of rennet-treated milk with added NaCas ≥0.4% (w/w) suggest that non-sedimentable casein on hydrolysis by chymosin forms into small aggregates that remain soluble (in suspension at low *g*-force, e.g., 3,000 *g*) but sediment increasingly as centrifugation force is ramped to 100,000 *g*. It is likely that these soluble 'floating' aggregates physically come between and impede, or block, the aggregation of the rennet-altered *para*-casein micelles (present in the skim milk), and thereby impair their ability to cohere into a continuous gel network.

The addition of NaCas reduced heat stability of the fortified milk at pH ≤6.6 (pH of maximum stability) and increased stability at pH 6.8 to 7.2 (pH region of minimum stability), with the effect becoming more pronounced with level of NaCas

added. The enhanced heat stability at the higher pH values concurs with the results of Cho and Singh (1999), who found that the heat stability of milk-fat emulsions (~2%, w/w, protein) in the pH range 6.4 to 7.1 at 140°C was significantly higher when the emulsions were stabilized with NaCas instead of SMP. The influence of NaCas on heat stability at pH 6.8 to 7.2 is likely to be associated with the interactive effects of its relatively low levels of total calcium, [Ca^{2+}], sedimentable Ca, lactose and NPN (Table 4.2) (O'Connell and Fox, 2003; Huppertz, 2016). The heat stability of fortified milk is likely to be enhanced on increasing the level of added NaCas owing to the commensurate reductions in the contents of lactose (Berg & van Boekel, 1994), total Ca (Sikand et al., 2010), Ca^{2+} (Huppertz, 2016) and whey protein-to-casein ratio (Fox & O'Connell, 2003). Simultaneously, heat stability is likely to be attenuated by the reduction in NPN, and, hence, urea (Muir & Sweetsur, 1977) and by the increase in non-sedimentable κ-casein (O'Connell & Fox, 2003).

It is generally considered that the minimum in the heat stability curve of bovine milk is due to the dissociation of κ-casein from the micelle to the serum where it forms soluble complexes with β-lactoglobulin, and possibly other whey proteins, via sulphydryl-disulphide interchange in the serum (Donato & Guyomarc'h, 2009). The resultant κ-casein-depleted micelle is more susceptible to calcium-, heat- and ethanol-induced aggregation (Singh & Fox, 1986). However, the current study indicated a positive correlation between minimum heat coagulation time (Figure 4.6b) and non-sedimentable κ-casein (as a proportion of total κ-casein in the fortified milk), which increased from ~15 to 40% as NaCas was increased from 0 to 0.8% (w/w); a similar relationship was found between non-sedimentable κ-casein and heat coagulation time at pH 6.8, 6.9 or 7.0 with the effect increasing with pH. This anomaly probably reflects again the interactive contribution of different factors to

heat stability of the fortified milk. Hence, the expected decrease in heat stability with increasing depletion of micellar κ-casein (O'Connell & Fox, 2003) upon increasing added NaCas, may be more than off-set by the positive effects of the concomitant reduction in lactose, [Ca^{2+}], total Ca and whey protein-to-casein ratio.

The increase in ethanol stability with pH concurs with trends from other studies (Mohammed & Fox, 1986). The positive effect of NaCas on ethanol stability at pH 6.4 is likely to be associated with the reductions in sedimentable casein, calcium and [Ca^{2+}] (Horne & Parker, 1981; Mohammed & Fox, 1986; Horne & Muir, 1990) and lactose content (Lin et al., 2016). However, why such an effect would occur at pH 6.4 only is unclear.

4.5. Conclusion

The rennet gelation, heat stability and ethanol stability characteristics of protein-fortified skim milk (4.1% protein, w/w) were significantly affected by the weight ratio of SMP-to-NaCas in the protein blend used in fortification. These effects coincided with a change in the ratios of sedimentable-to-non-sedimentable casein and Ca, as influenced by the differences in the Ca and P contents between NaCas and SMP. Hence, a low degree of mineralisation and Ca-to-P ratio would be conducive to the formation of more heat- and ethanol-stable beverages, but would be detrimental to rennet-induced gelation of recombined milk.

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Chapter 5

Effect of heat treatment, evaporation and spray drying during skim milk powder manufacture on the compositional and processing characteristics of reconstituted skim milk and concentrate

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Abstract

The effects of key manufacturing steps (heat treatment, evaporation and spray drying) during the manufacture of low- and high-heat skim milk powders (SMP) on the physico-chemical and processing characteristics of milk, and concentrates of varying total solids (TS) levels, prepared by reconstituting the milk powders, were evaluated. Milk heat treatment had the most pronounced effect, with an increase in severity of heat treatment from 72°C for 15 s to 120°C for 120 s, prior to evaporation resulting in higher heat coagulation time (HCT) at pH 6.3-6.6 and ethanol stability (ES) at pH 6.2-6.6, and a marked deterioration of rennet-induced coagulability. Increasing TS of the milk on reconstitution from 9.4 to 25% reduced HCT at pH >6.3 and ES at pH 6.6-7.0, increased ES at pH 6.2-6.4, and led to partial recovery of rennet-coagulability. The results highlight how heat treatment may be used to customize the functionality of SMP to different applications.

Key words: heat treatment, whey protein denaturation, skim milk powder, skim milk concentrate, functionality

5.1. Introduction

Apart from its use in formulated foods such as sauces, custards, ice-cream and processed cheese products, skim milk powder (SMP) is extensively reconstituted to skim milk with different levels of total solids (e.g., 9-30%), for applications such as milk-based beverages, condensed milks, and recombined milks for cheese or yoghurt manufacture (Gilles & Lawrence, 1981; Lagrange et al., 2015; IDF, 1999). SMP is classified as low-, medium- or high-heat SMP according to the heat treatment applied to skim milk prior to evaporation and drying (Martin et al., 2007). Typical heat treatments are 70-72°C for 15 s for low-heat SMP, and 120°C for 60-120 s, or 90°C for 300 s for high-heat SMP (Kelly et al., 2003). High-heat SMP is used as an ingredient in bakery, sweetened condensed milk, and confectionery products such as ultra-high temperature (UHT) treated recombined concentrated milk, toffee, caramel, fudge and milk chocolate (Aitken et al., 1999; Stewart et al., 2017). Low-heat powder is also used extensively in food formulation, including applications such as recombined milk for cheese manufacture, milk solids standardization in products such as cheese milk, yoghurt and fermented milk products (Patel et al., 2007).

For all types of SMP, the stages of manufacture include heat treatment of the milk, evaporation to ~45-50% total solids (TS) and spray drying to ~97% TS. Heat treatment, depending on the severity (temperature and time) and milk pH, affects the extent of whey protein denaturation, the binding of denatured whey protein to the casein micelle and the partitioning of components (salts, whey protein and caseins) between the serum and colloidal phases of milk (Donato & Guyomarc'h, 2009). These changes

affect milk processing characteristics such as rennet gelation (Guinee et al., 1997; Pomprasirt et al., 1998), acid-induced gelation (Vasbinder et al., 2003a), heat stability (Sievanen et al.,2008), syneresis of acid-induced and rennet-induced-milk gels (e.g., yoghurt, cheese), and can result in altered cheese texture and functionality (Rynne et al., 2004).

Studies on the impact of heat treatment on the ethanol stability (ES) of skim milk concentrates are scarce, though the separate effects of heat treatment (Horne & Parker, 1981; Mohammed & Fox, 1986) and concentration (Horne & Parker, 1983) have been investigated. ES is of relevance in alcoholic milk-based beverages (e.g., cream liqueur, egg-nog and coquito) as an indicator of the resistance of the milk protein to aggregation and, hence, emulsion stability. Martin et al. (2007) reported that the casein micelle sizes in low-, medium- and high-heat treated skim milk increased during evaporation to 45% TS, and remained high in high-heat SMP on reconstitution. Singh and Creamer (1991) found that the heat coagulation time (HCT) of concentrated milk (prepared by diluting evaporated milk to 20% TS) in the pH region 6.3 to 6.6 increased significantly on increasing severity of heat treatment from 72°C for 15 s to 120°C for 180 s. Similarly, an increase in heat treatment from 110°C for 120 s to 120°C for 180 s affected the heat stability of reconstituted milk (9.7% TS), as evidenced by a shift in the HCT/pH curve to lower pH and the concomitant increase in HCT at pH 6.5-6.6 and reduction at pH 6.8-7.1 (Singh & Creamer, 1991).

The objective of the current study was to evaluate the impact of heat treatment, evaporation and spray drying on the partitioning of milk proteins

and minerals between serum and colloidal phases, rennet gelation, HCT and ES of the resultant milk samples, and concentrates prepared by reconstitution of the SMP.

5.2. Materials and methods

5.2.1. Manufacture of low heat and high heat skim milk powder

Skim milk powder was manufactured at Moorepark Technology Limited (Cork, Ireland). Milk was separated at 55°C (Westfalia Model MM1254 Separator; Westfalia, Germany) and the skim milk (≤0.1% fat) was pasteurized using a plate heat-exchanger (APV Pasilac SSP pilot plant, APV DK 8600, Silkeborg, Denmark) at 72°C for 15 s (low heat, LH) or using a MicroThermics® pilot-scale tubular heat-exchanger (MicroThermics, Raleigh, NC, USA) at 120°C for 120 s (high heat, HH). The pasteurized skim milk was cooled directly to 4°C, held at 4°C overnight, heated to 50°C, stirred for 30 min, concentrated to 45% TS (Anhydro Falling Film Evaporator Type F, SPX Flow Technology Danmark A/S, Soeborg, DK-2860, Denmark) and spray-dried (Anhydro Spray Dryer, SPX Flow Technology Danmark A/S, Soeborg, Denmark) using centrifugal disc atomization at inlet and outlet air temperatures of 180 and 85°C, respectively. The resultant LH- and HH-skim milk powders were each produced on two separate occasions (trials), with both powder types being produced from the same milk on each occasion.

5.2.2. Preparation of skim milk samples

Samples taken during powder manufacture included: skim milk, heat-treated skim milk, evaporated skim milk (45% TS) and powder. Samples of low heat-treated skim milk, evaporated skim milk and powder are denoted as

LHSM, LHE and LHP, respectively, and the corresponding high heat-treated samples as HHSM, HHE and HHP, respectively (Table 5.1).

The LHE and HHE samples were diluted with distilled water at 25°C and stirred (Model RW16; IKA-Werke GmbH, Staufen im Breisgau, Germany) at 750 rpm for 30 min to give skim milk with 9.4% TS, denoted as LHE-SM and HHE-SM, respectively (Table 5.1). Skim milk samples (9.4% TS), denoted LHP-SM and HHP-SM, were also prepared by reconstitution of the LHP and HHP in distilled water. The powder was dispersed in distilled water (50°C), held in a water bath (50°C) while stirring at 750 rpm for 30 min and stored at 4°C for 22 h to allow hydration of the protein; prior to analysis, the reconstituted skim milk samples were warmed to 40°C and held for 30 min to reverse the cold-aging, and then cooled to 25°C for analysis (Dalgleish & Law, 1988).

5.2.3. Compositional analysis of skim milk and serum

Skim milk samples were assayed for TS and fat using the CEM SMART Trac II (CEM, Matthews, North Carolina, USA), lactose using the FOSS MilkoScan™ FT+ (Foss Electric A/S, Hillerød, Denmark) and ionic calcium [Ca^{2+}], using a sensION+ 9660C calcium combination ion selective electrode (Hach Lange, Barcelona, Spain), as described in Chapter 2 and 3.

Serum was prepared by ultracentrifugation of skim milk at 100,000 *g* at 25°C for 1 h and filtration of the supernatant, as described in Chapter 2. Skim milk and serum were analysed for total protein, non-casein nitrogen (NCN), non-protein nitrogen (NPN), calcium (Ca), phosphorus (P), and protein profile using reversed-

phase high performance liquid chromatography (RP-HPLC) using methods described previously in Chapter 2.

The analysis scheme used to isolate the different nitrogen (N) /protein fractions of the HH samples is shown in Figure 5.1a; the measurements performed on the different samples and the parameters derived are shown in Fig. 1b. The true protein content of the serum was calculated as the difference between total (crude) protein of serum and NPN (expressed as protein). Total serum casein was calculated as the product of true protein in the serum and casein as a proportion of true protein in the serum, as measured by RP-HPLC.

On pH adjustment of the serum to pH 4.6, serum-soluble casein (κ-, β-, α_s-caseins) and denatured whey protein, assumed to be complexed with κ-casein in the form of serum-soluble aggregates (Mollé, Jean & Guyomarc'h, 2006), precipitate. Hence, the total protein concentration of the pH 4.6-soluble filtrate corresponds to native whey protein and NPN. The concentrations of serum-soluble casein and denatured whey protein/κ-casein aggregates were, thus, calculated as the difference between the total protein content of the serum and that of the pH 4.6 soluble filtrate. The difference in concentration between that of the latter (serum-soluble casein and denatured whey protein/κ-casein aggregates) and the serum casein corresponds to denatured whey proteins contributing to serum-soluble aggregates. The equations used in the calculation of the different N fractions in the serum phase are below:

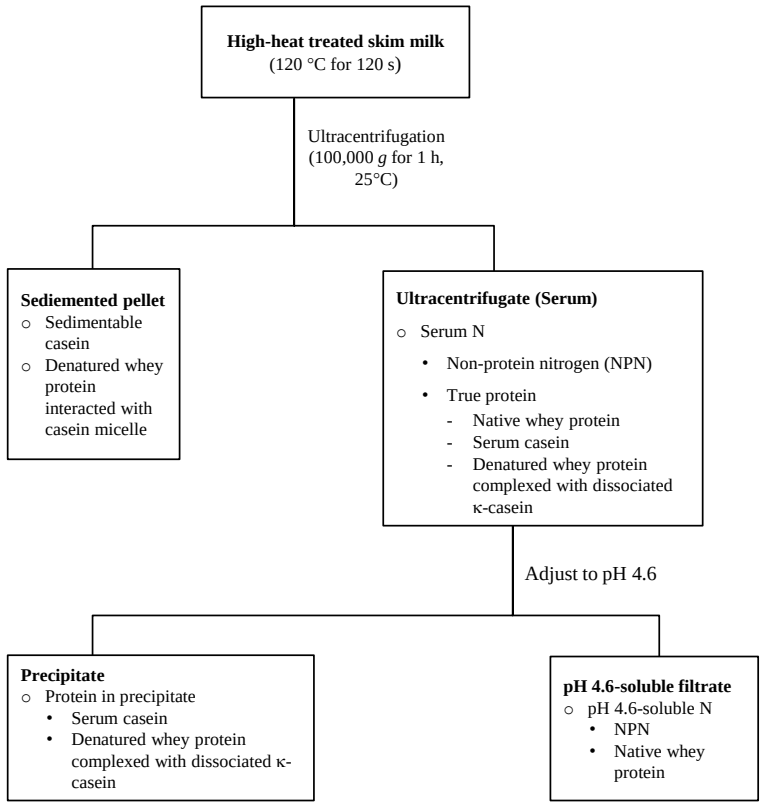
(1) True protein in serum (%, w/w) = total protein (%, w/w) – (NPN × 6.38) (%, w/w)

(2) Serum casein (%, w/w) = true protein (%, w/w) × casein (as % of true protein)

(3) Denatured whey protein complexed with dissociated κ-casein (%, w/w) = Total protein (%, w/w) – serum casein (%, w/w) – pH 4.6-soluble protein (%, w/w)

(4) Denatured whey protein complexed with κ-casein on the casein micelle (%, w/w) = Total denatured whey protein (%, w/w) – denatured whey protein complexed with dissociated κ-casein (%, w/w)

a



b

Sample	Measured parameter	Derived parameter
Skim milk samples: HHSM, HHE-SM, HHP-SM	Total protein, Non-casein N, NPN Protein profile	True protein Whey protein Casein
Serum	Total protein, NCN, NPN Protein profile	True protein (native whey protein, serum casein, serum-soluble denatured whey protein) Serum casein
pH4.6-soluble filtrate	TN	Native whey protein + NPN

Figure 5.1. a Flow chart showing the separation of high-heated skim milk samples (high-heat treated skim milk, HHSM; skim milk prepared by dilution of evaporated high-heat treated skim milk, HHE-SM; and skim milk prepared by reconstitution of high-heat skim milk powder, HHP-SM) into different nitrogen (N)/protein fractions, and b Analysis undertaken on the different fractions. Abbreviations: N, nitrogen; NPN, non-protein nitrogen; NCN, non-casein nitrogen; TN, total nitrogen.

5.2.4. Physico-chemical characteristics of skim milk samples

Casein micelle size, expressed as z-average (nm), and the apparent zeta potential of skim milk samples were determined using a Malvern Zetasizer Nanoseries Nano-ZS (Malvern Instruments Ltd, Malvern, UK), as described in Chapter 2. Casein hydration was measured by lyophilisation of the pellet obtained on ultracentrifugation, and expressed as g water /g sedimented casein (Chapter 2).

5.2.5. Preparation of skim milk concentrates

The LHP and HHP powders were reconstituted in distilled water for the preparation of concentrated milks with 9.4-25% TS, using a similar procedure to that used for the LHP-SM and HHP-SM skim milk samples. The concentrates from the LHP and HHP are denoted LHP-SMC and HHP-SMC, respectively (Table 5.1).

5.2.6. Calcium ion concentration of skim milk concentrates

The LHP-SMC and HHP-SMC samples, at 25% TS, were adjusted to pH values in the range 6.2 to 7.0, at 0.2 pH unit intervals. The [Ca^{2+}] of the pH-adjusted concentrates was immediately measured, as described in Section 2.3.

5.2.7. Rennet gelation of skim milk and skim milk concentrates

Samples of skim milk concentrates with 9.4-25% TS were adjusted to pH 6.55 and inoculated with chymosin (single strength Chy-Max® plus, 200 IMCU/mL; Chr. Hansen, Hørsholm, Denmark), which had been diluted 20-fold with distilled water, at a level of 0.103 mL/g protein. Milk samples were tested for rennet gelation properties at 31°C using dynamic low-amplitude strain oscillation rheometry in a controlled-stress rheometer (Carri-Med, type CSL^2_{500}, TA instruments, New Castle, DE, USA) at a strain of 0.025 and a frequency of 1 Hz, as described in Chapter 2.

The storage modulus, G′, was measured dynamically as a function of time over 1 h (G'_{60}); the gelation time (GT) was defined as the time for G′ to reach a threshold value of ≥0.2 Pa and the maximum curd firming rate as the maximum slope of the G′/time curve.

5.2.8. Heat coagulation time of skim milk and skim milk concentrates

Samples of skim milk (SM, LHSM, HHSM, LHE-SM, HHE-SM, LHP-SM and HHP-SM) and skim milk concentrates with 15-25% TS (LHP-SMC, HHP-SMC) were adjusted to pH values in the range from 6.2 to 7.2 at room temperature using HCl or NaOH. The HCT of the skim milk and skim milk concentrate samples was measured at 140 and 120°C, respectively, as described in Chapter 2. Preliminary trials indicated that skim milk concentrates with 15-25% TS were sometimes prone to instantaneous coagulation at 130 or 140°C depending on pH, while concentrates with ≥25% TS gelled/solidified instantly at temperatures ≥120°C.

5.2.9. Ethanol stability of skim milk concentrates

Skim milk concentrates with 9.4-25% TS were prepared by reconstitution of SMP and adjusted to pH values in the range 6.2 to 7.0 at 0.2 pH unit intervals. The ES was tested by blending 1 mL of sample with aqueous ethanol solutions of different concentrations (30-98%) while keeping the ethanol-to-protein ratio constant. The mixture of aqueous ethanol and sample was mixed for 30 s before inspection for visible flocculation (Chapter 2).

5.2.10. Statistical analysis

Data were analysed using a randomized complete block design, which incorporated the skim milk samples (SM, LHSM, HHSM, LHE-SM, HHE-SM, LHP-SM, HHP-SM) or skim milk concentrate (LHP-SMC and HHP-SMC) and 2

replicate blocks (samples from the 2 separate bathes of SMP or evaporated milk made on different days). Analysis of variance (ANOVA) was carried out using a general linear model (GLM) procedure of SAS 9.3 (SAS Institute, 2011) and the effects of treatment (stage of manufacture: heat treatment, evaporation and drying) and replicate on each response variable was determined. Tukey's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

Regression analysis was performed to investigate potential correlations between G'_{60} and TS in the skim milk concentrates.

5.3. Results

5.3.1. Gross composition of skim milk samples

The composition of the skim milk samples (SM, LHSM, HHSM, LHE-SM, HHE-SM, LHP-SM, HHP-SM; Table 5.1) is shown in Table 5.2. As expected, all samples had similar levels of TS, lactose, total protein, casein, NPN (% total N), total Ca and P. Increasing the heat treatment of the skim milk prior to evaporation led to a significant increase in whey protein denaturation from ~5% (72°C for 15 s) to 80% of total whey protein (120°C for 120 s; Table 5.2).

Table 5.1. Samples collected and analysed during manufacture of skim milk powder[1,2]

	Codes
Samples taken during manufacture of skim milk powder	
Skim milk (unheated)	SM
Low-heat treated skim milk	LHSM
High-heat treated skim milk	HHSM
Low-heat evaporated skim milk	LHE
High-heat evaporated skim milk	HHE
Low-heat skim milk powder	LHP
High-heat skim milk powder	HHP
Skim milk samples analysed	
SM	SM
LHSM	LHSM
HHSM	HHSM
Diluted LHE	LHE-SM
Diluted HHE	HHE-SM
Reconstituted LHP	LHP-SM
Reconstituted HHP	HHP-SM
Skim milk concentrates analysed	
Reconstituted LHP	LHP-SMC
Reconstituted HHP	HHP-SMC

[1]Skim milk was subjected to low-heat treatment (LH, 72°C for 15 s) or high-heat treatment (HH, 120°C for 120 s).
[2]The total solids content of skim milk samples was 9.4%, and that of skim milk concentrates was 9.4, 15, 20 or 25%.

Table 5.2. Composition of skim milk and serum.[1,2,3,4]

		Low-heat treatment (LH)			High-heat treatment (HH)		
Composition	SM	LHSM	LHE-SM	LHP-SM	HHSM	HHE-SM	HHP-SM
Skim milk							
Total solids (%, w/w)	9.39[a]	9.40[a]	9.30[a]	9.43[a]	9.38[a]	9.48[a]	9.50[a]
Lactose (%, w/w)	4.60[a]	4.57[a]	4.58[a]	4.59[a]	4.56[a]	4.58[a]	4.53[a]
Total protein (%, w/w)	3.91[a]	3.90[a]	3.90[a]	3.92[a]	3.90[a]	3.89[a]	4.06[a]
Casein (%, w/w)	3.09[a]	3.09[a]	3.09[a]	3.09[a]	3.09[a]	3.09[a]	3.09[a]
WP (%, w/w)	0.62[a]	0.62[a]	0.62[a]	0.62[a]	0.62[a]	0.62[a]	0.62[a]
Denatured WP (% total WP)	0[c]	4.78[b]	4.18[b]	5.44[b]	82.46[a]	81.70[a]	80.75[a]
Denatured WP associated with casein micelle (% total denatured WP)		ND	ND	ND	92.0[a]	85.6[a]	86.7[a]
NPN (% TN)	5.60[a]	5.97[a]	6.06[a]	5.85[a]	5.77[a]	6.07[a]	5.97[a]
Ionic Ca, [Ca^{2+}] (Normalized, % [Ca^{2+}] in SM)	100.0[a]	99.5[ab]	94.0[c]	97.3[b]	90.7[d]	81.2[e]	88.7[d]
Total Ca (mg/100 g)	124[a]	123[a]	122[a]	122[a]	124[a]	122[a]	126[a]
Total P (mg/100 g)	102[a]	100[a]	103[a]	105[a]	100[a]	103[a]	103[a]
pH	6.68[a]	6.68[a]	6.68[a]	6.69[a]	6.66[a]	6.69[a]	6.70[a]
Casein hydration (g water/g casein)	3.05[a]	3.09[a]	3.10[a]	3.02[a]	3.19[a]	3.05[a]	3.02[a]
Particle size (nm)	166[d]	167[cd]	176[bc]	179[b]	186[b]	209[a]	213[a]
Zeta potential (mV)	–22.4[a]	–22.9[a]	–20.6[a]	–24.0[a]	–22.8[a]	–22.8[a]	–22.3[a]
Skim milk serum							
Protein (%, w/w)	1.10[a]	1.11[a]	1.02[a]	1.09[a]	0.70[b]	0.71[b]	0.70[b]
Protein (% milk protein)	27.9[a]	28.3[a]	26.0[a]	27.4[a]	17.8[b]	18.2[b]	17.8[b]
Casein (%, w/w)	0.21[b]	0.25[b]	0.21[ab]	0.22[b]	0.42[a]	0.44[a]	0.42[a]
Casein (% milk casein)	6.79[b]	8.01[b]	6.93[ab]	6.95[b]	13.58[a]	14.16[a]	13.68[a]
Whey protein							
α-lactalbumin (% α-Lac in SM)	100.0[a]	98.9[a]	98.9[a]	95.5[a]	38.6[b]	24.1[b]	29.5[b]
β-lactoglobulin A (% β-Lg A in SM)	100.0[a]	100.0[a]	94.8[a]	96.4[a]	18.7[b]	21.6[b]	20.6[b]
β-lactoglobulin B (% β-Lg B in SM)	100.0[a]	100.0[a]	96.3[a]	97.2[a]	13.9[b]	16.1[b]	15.6[b]
Ca (mg/100 g)	45[a]	45[a]	29[b]	45[a]	30[b]	29[b]	31[b]
Ca (% milk Ca)	35.9[a]	37.1[a]	23.9[b]	37.2[a]	24.3[b]	24.1[b]	24.9[b]
P (mg/100 g)	47[a]	50[a]	30[b]	50[a]	32[b]	34[b]	30[b]
P (% milk P)	46.2[a]	49.9[a]	29.0[b]	47.2[a]	31.6[b]	32.4[b]	29.1[b]

[1]Samples, as defined in Table 1 include: unheated skim milk, low-heat treated skim milk (LHSM), skim milk prepared by dilution of evaporated low-heat treated skim milk (LHE-SM) or by reconstitution of low-heat skim milk powder (LHP-SM); the corresponding samples from high-heat treated skim milk include HHSM, HHE-SM, and HHP-SM. Skim milk serum was obtained by ultracentrifugation at 100,000 *g* for 1 h at 25°C.

[2]Presented data are the mean values of duplicate trials; values within a row not sharing a common lower-case superscript letter differ significantly ($P < 0.05$).
[3]The ionic Ca content of SM was set at 100, and the values for all other samples as a percentage of the value in SM.
[4]ND, not determined. NPN, non-protein nitrogen; TP, total protein; TN, total nitrogen; WP, whey protein, [Ca^{2+}], ionic calcium; Ca, calcium; P, phosphorus; α-lactalbumin, α-Lac; β-lactoglobulin, β-Lg A; β-lactoglobulin B, β-Lg B.

The concentration of ionic Ca, [Ca^{2+}], in the unheated skim milk from Trials 1 (2.1 mM) and 2 (~5.0 mM) differed markedly. The values, though very different, reflect the range reported in the literature for bovine milk (~1-5 mM) (White & Davies, 1958; Kelly, Keogh, O'Keeffe & Phelan, 1982). Hence, the value of [Ca^{2+}] was normalized to 100 for the skim milk in both Trials 1 and 2, to facilitate statistical analysis. HH treatment led to a significant reduction in [Ca^{2+}], but low heat treatment had no effect, as reflected by the similar [Ca^{2+}] in the SM and LHSM samples (Table 5.2). During the manufacture of both LHP and HHP, evaporation led to a reduction in [Ca^{2+}], while drying resulted in restoration to a level equal to that of the LHSM and HHSM samples, respectively. The mean [Ca^{2+}] value of the HHSM, HHE-SM and HHP-SM were significantly lower than those of the corresponding samples of LHSM, LHE-SM and LHP-SM (Table 5.2).

5.3.2. Physico-chemical properties of skim milk samples

All skim milk samples showed a mono-modal particle size/number distribution. Casein micelle size increased significantly during the manufacture of both LHP and HHP, with the increase occurring during evaporation for the former, and increased during milk heat treatment and evaporation for the latter (Table 5.2). Particle sizes for the LHSM, LHE-SM and LHP-SM were significantly lower than those of the corresponding HHSM, HHE-SM and HHP-SM. The zeta potential and hydration of all skim milk samples ranged from -20.6 to -24 mV and 3.02 to 3.19 g

water/g casein, respectively, and were not significantly affected by heat treatment, evaporation or drying.

5.3.3. Composition of the sera from skim milk samples

The concentrations of serum β-lactoglobulin A (β-Lg A), β-lactoglobulin B (β-Lg B) and α-lactalbumin (α-Lac) in the HHSM, HHE-SM and HHP-SM milk from the milk heated at 120°C were ~19-22, 14-16, and 24-39%, respectively, of the level in the unheated skim milk, SM; the corresponding levels in the LHSM, LHE-SM and LHP-SM samples were ~95-100, 96-100, and 96-99%, respectively. This result is consistent with the increase in whey protein denaturation on intensifying milk heat treatment (Table 5.2). Evaporation and drying did not induce denaturation of whey proteins during the preparation of the SMP, as evidenced by the similar levels of whey proteins in the serum (expressed as a % of the unheated SM) in the heated skim milk, evaporate and reconstituted SMP for both the LH and HH treatment.

The concentration of serum caseins, α_s-, β- or κ-casein, expressed as % of the corresponding casein in skim milk, was not affected by heat treatment (72°C for 15 s), evaporation or drying during the manufacture of LHP, as indicated by similar values in the SM, LHSM, LHE-SM and LHP-SM. In contrast, heat treatment (120°C for 120 s) during the manufacture of HHP resulted in significant increases in the levels of serum casein and κ-casein (Table 5.2, Figure 5.2a). For both the LH and HH milk samples, the level of serum κ-casein (% κ-casein in milk) was higher than that of serum β- or α_s-casein (Figure 5.2a). Nevertheless, owing to the different concentrations of the individual caseins in milk, the proportions of different serum

caseins, expressed as % of total serum casein, were not significantly affected by heat treatment, evaporation or drying during the manufacture of the LHP or HHP (Fig. 2B)

In contrast to serum casein, the concentrations of serum Ca and P decreased significantly during the manufacture of HHP, as seen on comparing the SM and HHP-SM samples (Table 5.2); the reduction was observed entirely during the heating step (120°C for 120 s), with no further reduction during evaporation and drying. In the manufacture of LHP, serum Ca and P were not affected by heat treatment (72°C for 15 s), decreased during evaporation, and increased during drying. Nevertheless, the levels of serum Ca and P in the LHP-SM and SM were similar, indicating no overall influence during the manufacture of LHP. Consequently, serum Ca and P levels in the LHP-SM were significantly higher than that of the HHP-SM.

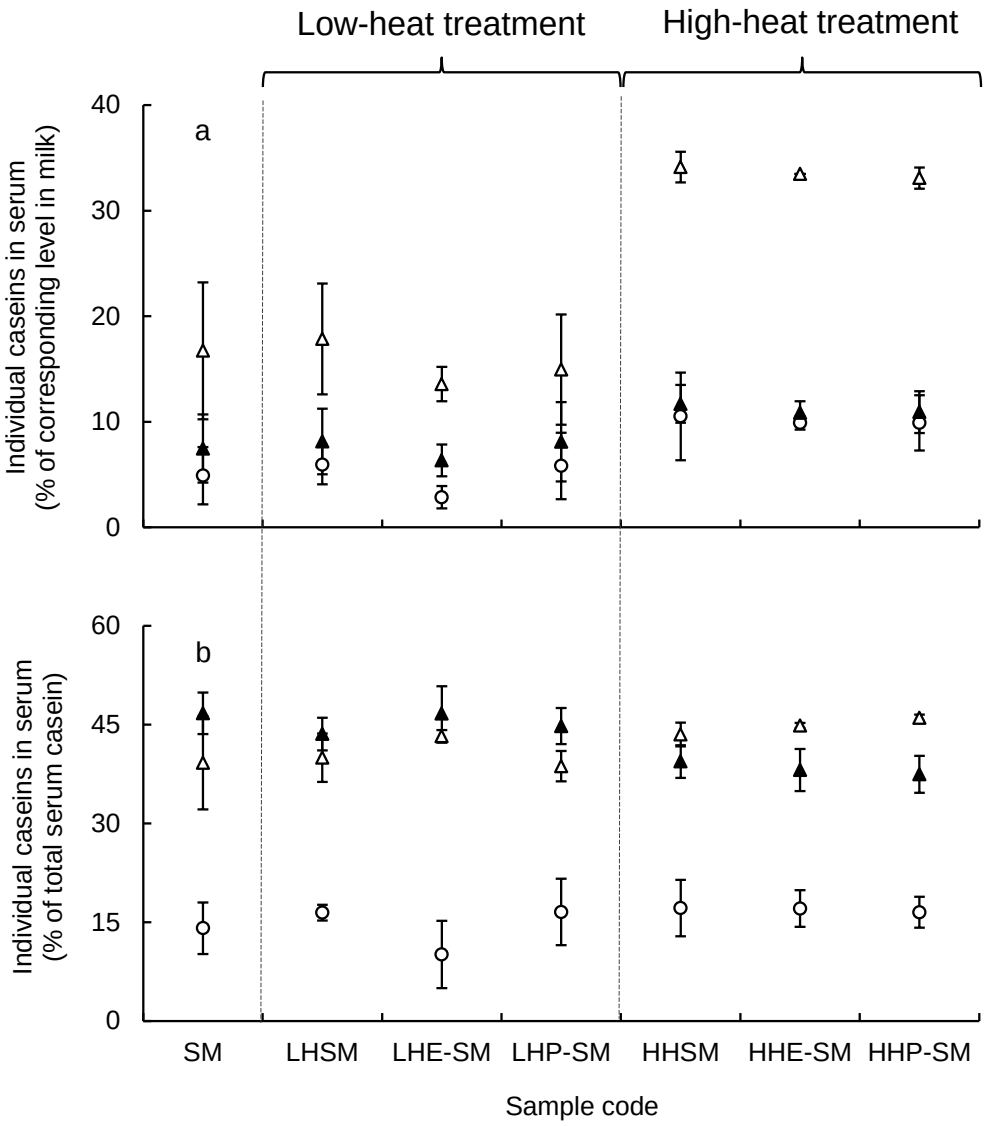


Figure 5.2. Concentration of caseins in serum prepared by ultracentrifugation of skim milk samples at 100,000 *g* for 1 h at 25°C: α_{s1} + α_{s2}-casein (○), β-casein (▲) and κ-casein (△). Samples, as defined in Table 5.1, include unheated skim milk (SM), low-heat treated skim milk (LHSM), skim milk prepared by dilution of evaporated low-heat treated skim milk (LHE-SM) or by reconstitution of low-heat skim milk powder (LHP-SM); the corresponding samples from high-heat treated skim milk, i.e., HHSM, HHE-SM, and HHP-SM. Data presented are the means of duplicate batches of each treatment; error bars represent the standard deviation of the mean.

5.3.4. Calcium ion content of skim milk concentrates

The $[Ca^{2+}]$ of the LH concentrates (LHP-SMC) at pH 6.2-7.0 increased slightly, but significantly, with increasing % TS; an opposite effect was found for the

HH concentrates (HHP-SMC) (Figure 5.3a). The [Ca^{2+}]/casein ratio decreased with TS, with the magnitude of the difference between the low (9.4%) and high (25%) TS concentrate decreasing as pH increased (Figure 5.3b). For both concentrates, the [Ca^{2+}] decreased with increasing pH (Figure 5.3a).

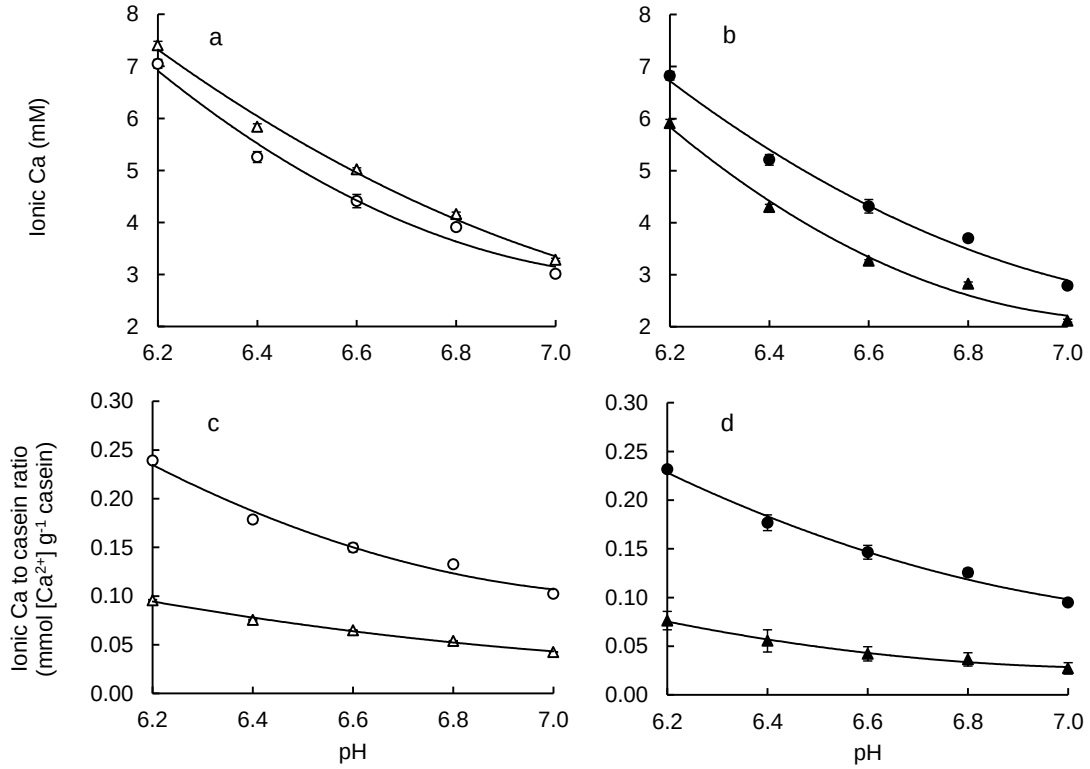


Figure 5.3. Changes in concentration ionic calcium, [Ca^{2+}], and [Ca^{2+}]:casein ratio as a function of pH for skim milk concentrates with total solids content of 9.4% (○,●) or 25% (△,▲), prepared by reconstituting low-heat skim milk powder (a, c) or high-heat skim milk powder (b, d).

5.3.5. Rennet gelation of skim milk and skim milk concentrates

The changes in gel strength, G', of the LH- and HH-skim milk samples with time after rennet addition are shown in Figures 5.4a and 5.4b, respectively. The values of G'_{60} of LH samples from Trial 2 were notably higher than those from trial 1,

an effect most likely associated with higher concentrations of protein and [Ca^{2+}] of the SM in Trial 2. The protein content of the skim milk from Trials 1 and 2 was 3.77 and 4.05%, respectively (data not shown).

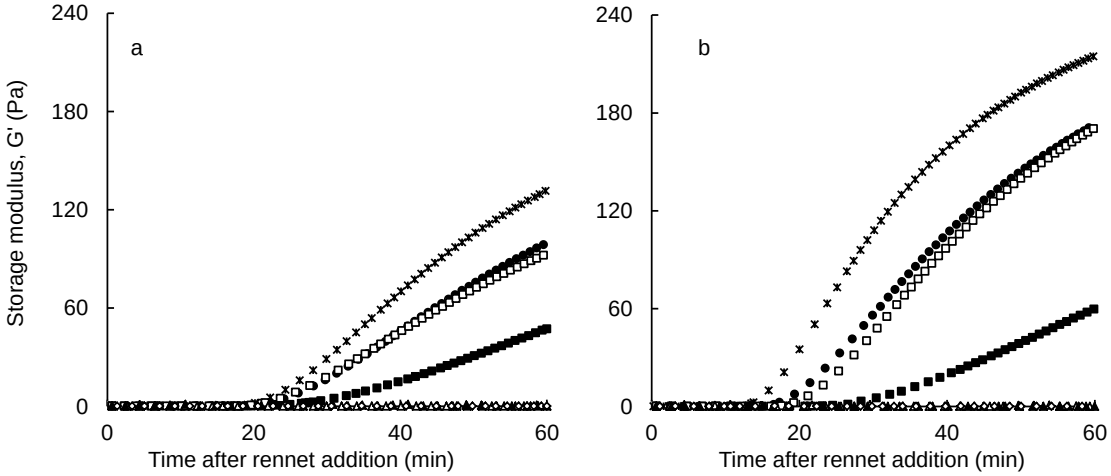


Figure 5.4. Development of storage modulus, G′, in rennet-treated skim milk samples from duplicate batches: Trial 1 (a) and Trial 2 (b). Samples, as defined in Table 5.1, include unheated skim milk (∗), low heat-treated skim milk (●), and skim milk prepared by dilution of evaporated low-heat treated skim milk (■) or by reconstitution of low-heat skim milk powder (□); high-heat treated skim milk (△), and skim milk prepared by dilution of evaporated high-heat treated skim milk (▲) or by reconstitution of high-heat skim milk powder (◇).

G′ deteriorated during the heat treatment and evaporation stages of LHP manufacture, but recovered during drying, as shown by the similar magnitude of G′ with time in the LHSM and LHP-SM milk samples. HH treatment irreversibly impeded rennet coagulability, as indicated by the failure of the HHSM, HHE-SM, HHP-SM to undergo gelation.

Increasing TS was parallelled by a significant reduction in gelation time and increases in gel-firming rate and G'_{60} of both the LHP-SM and HHP-SM samples (Figure 5.5a-d). G′ increased with increasing TS in the LHP-SM samples, with regression analysis indicating a power law dependency of G'_{60} on TS (LH: r = 0.98, n

= 8), where G'_{60} = (total solids)^n, and the exponent *n* was 2.4 (Figure. 5.5e). The increase in G' of the LHP-SMC samples with TS reflects the increase in the concentration of casein contributing to the structure of the calcium phosphate *para*-casein gel network, and the attendant increase in its stress-bearing capacity. While there was no improvement in the rennet coagulability of the HHP-SMC samples on increasing TS from 9.4 to 15%, G' increased linearly at a rate of ~8.5 Pa/g TS with a further increase in TS from 15 to 20-25% (Figure 5.5f). Hence, while the rennet gelation characteristics of the reconstituted LH- and HH-powders improved with increasing TS concentration, the rate of increase in G'_{60} was markedly lower in the latter than the former.

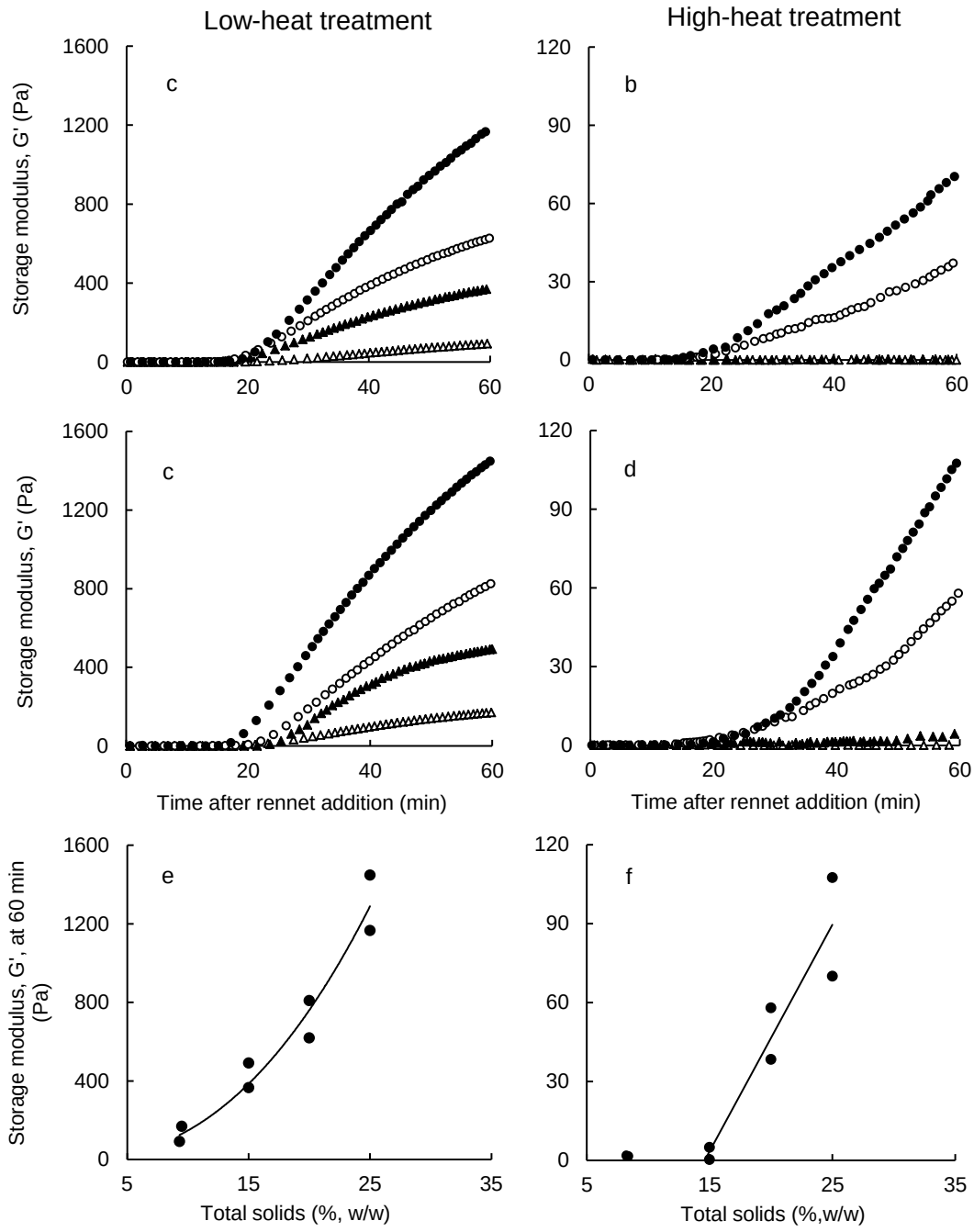


Figure. 5.5. Development of storage modulus, G′, in rennet-treated skim milk concentrates with 9.4 (△), 15 (▲), 20 (○) or 25 (●) % total solids. The concentrates were prepared by reconstituting low-heat (a, c) or high-heat (b, d) skim milk powder from duplicate batches: Trial 1 (a, b) and Trial 2 (c, d). Storage modulus at 60 min, $G'_{60,}$ as a function of total solids level for concentrates prepared from low-heat (e) or high-heat (f) skim milk powder; presented data for G'_{60} in both (e) and (f) is from Trials 1 and 2.

5.3.6. Heat stability of skim milk and skim milk concentrates

The HCT/pH curves for SM, LHSM and HHSM samples are shown in Figure 5.6a-d. All curves displayed the typical type A profile, with a maximum (HCT_{max}) and a minimum (HCT_{min}). The processing steps during the manufacture of LHP had little, or no, effect, as seen from the similar profiles of the SM, LHSM, LHE-SM and LHP-SM samples. In contrast, HH treatment during the manufacture of HHP reduced the pH of HCT_{max} by 0.1 and broadened the pH region of HCT_{min}, as observed by comparing the SM and HHSM samples. Otherwise, evaporation and drying during the manufacture of HHP had little impact on the heat stability characteristics of skim milk, as seen by the similarity of the HCT/pH profiles for the HHSM, HHE-SM and HHP-SM samples. High-solids recombined milks, which generally have relatively low pH compared with skim milk, are frequently subjected to heating (e.g., pasteurization and sterilization); consequently, the HCT/pH profile of reconstituted skim milk with varying TS is of interest.

The HCT/pH profiles of milk samples with TS of 9.4 to 25% at 120°C are shown in Figure 5.7a-d. The HCT of the samples from Trial 2 was lower than that of Trial 1 at corresponding pH values, probably because of the slightly higher protein content and [Ca^{2+}] of milk from Trial 2. Nevertheless, the reduction in HCT with TS was similar for both trials. At 9.4% TS, the HCT of HHP-SM showed a typical type A profile, with a distinct HCT_{max} at 6.5 and HCT_{min} at 6.7-6.8, whereas that of the LHP-SM increased continuously on increasing pH to pH 6.9 and then decreased slightly as pH was further increased to 7.0. Compared to the LHP-SM (9.4% TS), the HCT of the HHP-SM skim milk was 35 to 100 min higher than that of the LHP-SM at pH 6.3-6.5 and ~20 to 34 min lower at pH 6.7-6.9.

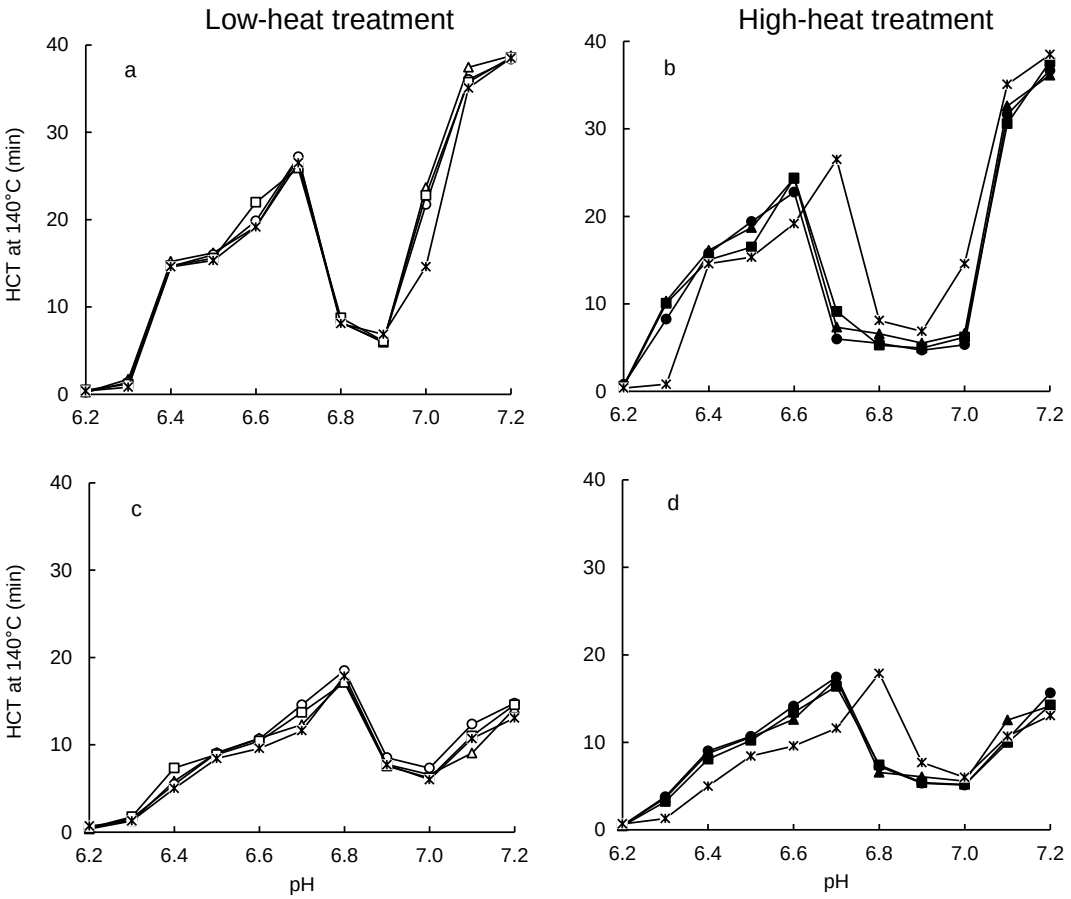


Figure 5.6. Heat coagulation time, HCT, at 140°C as a function of pH for skim milk samples, as defined in Table 5.1: unheated skim milk (∗); low-heat treated skim milk (△); high-heat treated skim milk (▲); skim milk prepared by dilution of evaporated low-heat treated skim milk (○) or high-heat treated skim milk (●); and skim milk prepared by reconstitution of low-heat skim milk powder (□) or high-heat skim milk powder (■). Samples were obtained from duplicate batches, Trial 1 (a, b) and Trial 2 (c, d).

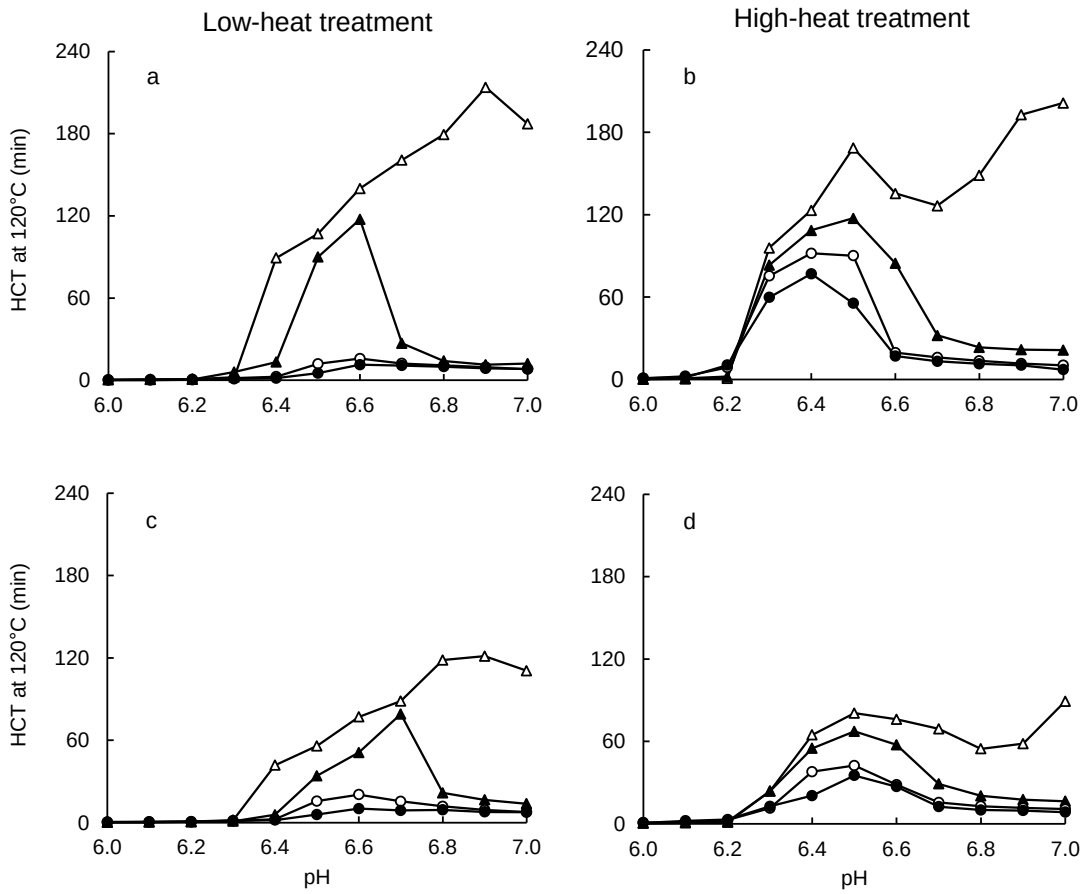


Figure. 5.7. Heat coagulation time, HCT, at 120°C as a function of pH for skim milk concentrates with 9.4 (△), 15 (▲), 20 (○) or 25 (●) % total solids. The concentrates were prepared by reconstitution of low-heat (a, c) or high-heat (b, d) skim milk powder. Samples were obtained from duplicate batches of skim milk powder, Trial 1 (a, b) and Trial 2 (c, d).

The HCT of both the LHP-SMC and HHP-SMC samples at pH values ≥6.4 decreased on increasing TS from 9.4 to 25% (Figure 5.7a-d). A major difference between the LHP-SMC and HHP-SMC samples was the higher HCT of HHP-SMC concentrates (20 and 25% TS) at pH values 6.3-6.6. Hence, while the HCT of the LHP-SMC with 20-25% TS was very low (<10 min) at all pH values, that of the corresponding HHP-SMC was quite high in the pH region 6.3-6.6, e.g., 90 (Trial 1) and 77 min (Trial 2) at pH 6.4 and 90 (Trial 1) and 55 min (Trial 2) at pH 6.5 (Figure 5.7b). The results clearly indicate that increasing the severity of the heat treatment of

the skim milk prior to powder manufacture enhances the heat stability of high-solids skim milk concentrates (20-25% TS), or conversely enables reconstitution of skim milk powder to higher TS while retaining adequate heat stability at pH 6.3-6.5 during thermal processing of recombined milks.

5.3.7. Ethanol stability of skim milk concentrates

The ethanol concentration/pH profiles of the skim milk concentrates (LHP-SMC and HHP-SMC) samples with TS ranging from 9.4 to 25% are shown in Figure 5.8a-d. The stability of all samples to ethanol increased with increasing pH. The ES of the HHP-SMC samples was numerically higher than that of the corresponding LHP-SMC samples at pH ≤6.6, but similar at pH 6.8 and 7.0; however, the magnitude of the differences between the corresponding LH and HH samples in the pH region 6.2–6.6 was significant ($P < 0.05$) at some pH values only, as indicated by different lower-case superscripts (a, b) (Figure 5.8a-d). The ES of the LHP-SMC and HHP-SMC samples at pH values 6.2 and 6.4 increased with TS, while the ES at pH 6.6–7.0 decreased (Figure 5.8e, f).

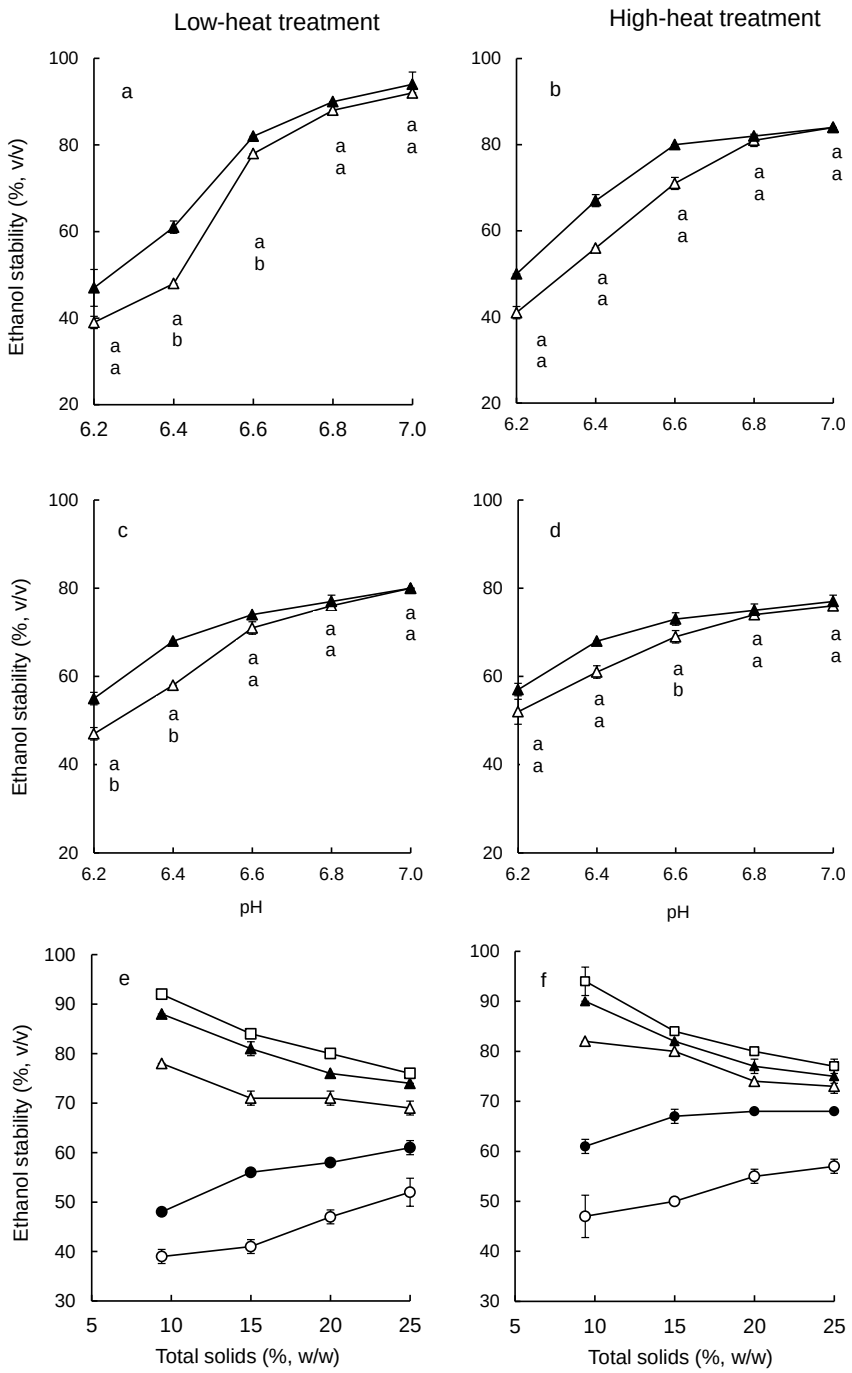


Figure 5.8. (a-d) Ethanol stability as a function of pH for skim milk concentrates with 9.4 (a), 15 (b), 20 (c) or 25 (d) % total solids; the concentrates were prepared by reconstituting low-heat (△) or high-heat (▲) skim milk powder. (e-f) Ethanol stability of concentrates as a function of total solids at pH 6.2 (○), 6.4 (●), 6.6 (△), 6.8 (▲) and 7.0 (□); the concentrates were prepared by reconstituting low-heat (e) or high-heat (f) skim milk powder. Presented data are the means of duplicate batches of each treatment; error bars represent the standard deviation of the mean.

5.4. Discussion

The manufacture of SMP involves heat treatment, evaporation and drying. The separate and combined effects of each step on the properties of reconstituted milk prepared from the SMP were evaluated in the current study. The severity of the heat treatment of milk prior to evaporation and drying had a major influence on the properties of reconstituted milk prepared from the powder. The level of heat treatment affected the partitioning of caseins, whey protein and minerals between the serum and the sedimented phase, rennet gelation, HCT and ES. By comparison, the evaporation and drying stages of skim milk powder manufacture had little, or no, effect on the characteristics of reconstituted milk. Hence, the properties of reconstituted skim milk are quite similar to those of the unheated skim milk for low heat SMP.

Increasing the severity of heat treatment of the skim milk prior to evaporation led to a significant increase in whey protein denaturation and casein micelle size, and reductions in the concentrations of whey proteins, Ca and P in the serum. The reduction in serum Ca and P suggests that calcium phosphate which precipitates during high heat treatment does not fully re-solubilise on cooling (Singh et al., 1996; van Hooydonk et al., 1987).

In contrast to the trend for serum whey protein, the concentration of serum casein increased significantly with HH treatment, mainly as a consequence of an increase in the concentration of serum κ-casein (% total κ-casein). This increase in serum κ-casein confirms the results of previous studies showing a significant increase in the extent of dissociation of κ-casein from the micelle into the serum as the temperature at heat treatment increased, e.g., from 60 to 120°C (Anema & Li,

2015; Ménard et al., 2005). It has been shown that the dissociated κ-casein interacts with denatured whey protein in the serum to form serum-soluble aggregates or particles (Donato & Guyomarc'h, 2009; Ménard et al., 2005; Mollé et al, 2006). Using a combination of chymosin-induced precipitation and capillary electrophoresis, Vasbinder et al. (2003a) determined the proportions of β-Lg and α-Lac that complexed with dissociated κ-casein (in the serum) and non-dissociated κ-casein (on the casein micelle) as the pasteurization temperature was increased from 70 to 90°C (for 10 min) at native pH; the level of β-Lg denaturation increased from ~2 to 95% of total β-Lg, and the level of serum casein increased from <5 to 10 % of total casein. Simultaneously, the proportions of total β-Lg involved in the formation of serum-soluble aggregates or associated with the casein micelle increased from ~2 to 25% or 1 to 65% of total, respectively (Vasbinder et al., 2003a). In the current study, the proportion of denatured whey protein that interacted with dissociated κ-casein was estimated at ~14% of total denatured whey protein in the HH-SMP; this estimate was based on the difference between the whey protein content of the HH-SMP serum and the filtrate obtained on pH-adjustment of the serum to pH 4.6. The interaction of most of the denatured whey protein (~86%) with the casein micelle was supported by the significantly higher casein micelle size in the HH skim milk samples. Likewise, Martin et al. (2007) reported progressive increases in whey protein denaturation and the hydrodynamic diameter of the casein micelle on increasing milk heat treatment from 79°C for 5 s to 90°C for 30 s or 120°C for 120 s.

Rennet gelation properties deteriorated significantly with HH treatment of the skim milk, and only partially recovered on increasing the TS of the reconstituted HHP to 25%. The adverse effect of heat treatment is likely to ensue from the

associated increase in the level of serum soluble κ-casein/β-Lg aggregates (Kethireddipalli & Dalgeleish, 2010; Vasbinder et al., 2003b) and reduction in [Ca^{2+}] in the HHSM (Sandra et al., 2012; Singh et al., 1988). Though the κ-casein in the κ-casein/β-Lg aggregates is hydrolysed by rennet, the aggregates, nevertheless, remain soluble following rennet-treatment and may impede the knitting of the *para*-casein micelles into a gel network continuum (Mollé et al., 2006). Various studies have shown that the hydrolysis of κ-casein in milk is unaffected by increasing heat treatment from 70 to 90°C for 10 min (Kethireddipalli & Dalgleish; 2010; Mollé et al., 2006; Vasbinder et al., 2003b). Rennet coagulability deteriorated during evaporation of the low-heat treated skim milk, as demonstrated by the significantly lower G'_{60} and GFR_{max} of the milks prepared by dilution of the LH evaporated milk (LHE-SM) compared to the LHSM. This was associated with a reduction in the serum concentration of [Ca^{2+}] in the LHE-SM (Table 5.2), probably because the time between concentrate dilution and measurement of rennet gelation (30-45 min) was insufficient to allow restoration of equilibrium between insoluble and soluble calcium (Chandrapala et al., 2010). This is corroborated by the similar [Ca^{2+}] and the rennet-gelation behaviour of the LHSM and the LHP-SM (Table 5.2); following reconstitution of the powder, the LHP-SM was held at 4°C for ~22 h.

HH treatment of skim milk before evaporation reduced the pH of HCT_{max}, broadened the HCT_{min} region, and increased HCT at pH values 6.3 to 6.6; this effect became more pronounced in skim milk concentrates as the TS was increased from 9.4 to 25%. These effects in the HH milk were parallelled by an increase in the proportion of denatured whey protein (86%) interacted with the casein micelle and a reduction in [Ca^{2+}]. It has been suggested that the interaction limits the dissociation of κ-casein during HCT measurement (Singh & Creamer, 1991). The role of ionic

calcium has been confirmed by Sievanen et al. (2008), who reported that the addition of 5 mM $CaCl_2$ to milk, before or after preheating (90°C for 10 min), significantly reduced the HCT. The HCT of both LHP-SMC and HHP-SMC decreased markedly on increasing TS from 9.4 to 25%. This trend, which concurs with results of Singh and Creamer (1991), has been attributed to the increases in volume fraction of casein and heat-induced acidification, associated with the thermal degradation of lactose to organic acids, dephosphorylation of casein, and to the precipitation of calcium phosphate (O'Connell & Fox, 2003).

At all TS levels (9.4-25%), the ES of the HHP-SMC concentrates in the pH range 6.2-6.6 was higher than that of corresponding LHP-SMC concentrates, an effect most likely due to the lower [Ca^{2+}] of the former (Horne & Parker, 1981; Mohammed & Fox, 1986). ES as a function of TS of both the LHP-SMC and HHP-SMC concentrates increased at pH 6.2 and 6.4 but decreased at pH 6.6-7.0. The pH-dependence of ES on TS may be related to the effect of pH on [Ca^{2+}] and, in particular, the [Ca^{2+}]/casein ratio. It is feasible that the difference in [Ca^{2+}] between the low and high TS concentrates is sufficiently large to influence ES in the pH region 6.2-6.4 but not at pH 6.6-7.0. As the relative contribution of the lower [Ca^{2+}]/casein ratio to the ES diminishes with increasing pH, the full effect of increasing TS, and hence casein, becomes apparent at higher pH values. Likewise, Horne and Parker (1983) found that the ES of concentrates from unpasteurised skim milk at pH 6.7-7.0 deteriorated progressively on increasing TS from ~9 to 23%. Based on model experiments, Horne and Parker (1983) concluded that the negative effect of increasing TS on ES at pH >6.7 was due to the increase in chloride content, and hence ionic strength. It was hypothesised that higher ionic strength resulted in a shift in calcium-citrate equilibrium, which favoured a higher [Ca^{2+}] concentration,

and hence lower ethanol stability, in high-solids concentrates. Nevertheless, the results of the current study showed that the [Ca^{2+}]/casein ratio decreased with increasing pH.

5.5. Conclusion

The changes in the partition of milk components (minerals and proteins), between the casein micelle and serum, and processing characteristics of milk at the different stages of manufacture of low-heat and high-heat skim milk powder were investigated. Increasing heat treatment of skim milk from 72°C for 15 s to 120°C for 120 s resulted in higher levels of whey protein denaturation, serum casein, serum κ-casein as a proportion of total κ-casein, and casein micelle size, and in lower concentrations of ionic calcium, and of serum calcium and phosphorous in skim milk and reconstituted skim milk. These changes were parallelled by marked deterioration in rennet coagulability, and increases in HCT at pH 6.3-6.6 and ES at pH 6.2 and 6.4. Increasing the TS level from 9.4 to 25% in skim milk concentrates, prepared by reconstitution of the skim milk powder, coincided with lower HCT at pH 6.3–7.0, lower ES at pH 6.6–7.0, higher ES at pH 6.2 and 6.4, and a partial recovery of rennet coagulability in the case of skim milk concentrates prepared from high-heat SMP (at TS ≥20%). The findings indicate how the intensity of heat treatment during the manufacture of skim milk powder can be altered to modulate the functionality of reconstituted skim milk and its suitability in different applications, e.g., recombined milk cheese or UHT-based milk beverages.

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Chapter 6

Altering the physico-chemical and processing characteristics of high heat-treated skim milk by increasing the pH prior to heating and restoring after heating

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Abstract

Skim milk was pH-adjusted from 6.6 to 7.5, high heat treated (HHT, 95°C for 2 min) or held unheated for 1 h, re-adjusted to pH 6.6, and analysed. HHT at pH 6.6 resulted in denaturation of ~67% of total whey protein, partial association of denatured whey protein with the casein micelle, an increase in casein micelle size, and reductions in concentrations of serum casein, Ca and P. These changes were paralleled by a marked deterioration in rennet coagulability, higher ethanol stability in the pH range of 6.2-6.6 ($P < 0.05$), and a reduction in the pH of maximum heat coagulation time (HCT) ($P < 0.05$). Increasing the pH before heat treatment led to increases in casein dissociation and the concentrations of κ-casein and denatured whey protein in the serum, and a reduction in casein micelle size ($P < 0.05$). Simultaneously, HCT at pH 6.6-6.7 and 7.2 increased significantly.

Key words: Skim milk, biochemical changes, high heat treatment, pH at heating, rennet gelation, heat stability, ethanol stability

6.1. Introduction

Milk, or recombined milk, is subject to high heat treatment (e.g., ≥80°C for 2-10 min) during the manufacture of medium- and high-heat milk powders, many dairy beverages (e.g., recombined evaporated milk, ultra-high temperature treated milk, infant milk formula, flavoured milks) and fermented milk products such as yoghurt and fresh acid-curd cheese (Sanderson, 1999). High-heat treatment (HHT) enhances the heat stability of reconstituted skim milk powder, extends the shelf-life of dairy beverages (Sharma et al., 2012) and promotes the development of viscosity and structure in fresh fermented milk products such as yoghurt and some fresh-fermented cheese products such as cream cheese and thermo Quark (Farkye, 2017; Guinee, 2016; Hinrichs, 2001).

HHT of milk at pH 6.5-6.7 (Anema et al., 2011; Vasbinder & de Kruif, 2003) leads to denaturation of a large proportion (e.g., >50%) of the whey protein, and its interaction with κ-casein on the surface of the casein micelle *via* hydrophobic bonding and thiol-disulphide interchange (Donato & Guyomarc'h, 2009; Guyomarc'h et al., 2003). Simultaneously, some of the serum calcium and phosphorus precipitate and associate also with the casein micelle (Schreiber, 2001). The denaturation of whey proteins and precipitation of calcium phosphate coincides with an increase in casein micelle size (Anema & Li, 2003a; Martin et al., 2007), enhanced acid gelation (higher viscosity) and a deterioration in the rennet coagulability (Donato & Guyomarc'h, 2009). The negative effect of HHT on rennet gelation at pH values close to native pH has been attributed to the presence of the denatured whey protein-κ-casein layer on the micelle which provides a steric hindrance to close approach and fusion of *para*-casein micelles (Schreiber, 2001). Mollé et al. (2006) demonstrated that HHT does not affect the hydrolysis of κ-casein, as measured by high-performance liquid chromatography coupled with mass spectrometry. However, it is likely that the heat-induced reduction in the concentration of ionic Ca ($[Ca^{2+}]$), and its slow solubilisation and re-equilibration on cooling

(Chandrapala et al., 2010), are also contributory factors (McMahon et al., 1984; Sandra et al., 2012).

As the pH at heating is increased, for example from 6.5 to 7.1, κ-casein dissociates increasingly from the micelle and complexes with denatured whey proteins in the serum to form serum-phase aggregates (Singh & Fox, 1985; Anema et al., 2007; Anema et al., 2011; Donato & Guyomarc'h, 2009; Ménard et al., 2005; Vasbinder & de Kruif, 2003). Vasbinder and de Kruif (2003) reported that increasing the pH of milk from 6.55 to 6.9 during HHT at 80°C for 10 min led to a slight improvement in the rennet coagulability, as measured at pH 6.7. In contrast, Ménard et al. (2005) found that an increase in the pH of milk from 6.6 to 7.1 during HHT (90°C for 30 s) led to an increase in rennet gelation time (measured at pH 6.5) and a reduction in gel firmness. An opposite effect was observed on increasing the pH before heating from 7.1 to 7.6 or 8.1, with milk high heat-treated at pH 7.6 or 8.1 having higher gel firmness than the corresponding milk heated at 6.6. Similarly, Anema et al. (2011) found that increasing the pH of milk from 6.55 to 7.1 prior to HHT at 90°C for 30 min impaired rennet gelation. The inter-study discrepancy for the effect of pH (6.55-7.1) before HHT on rennet coagulation may relate to differences in the experimental conditions used, such as extent of whey protein denaturation, the temperature to which the heated milk samples were cooled, the holding time (2-12 h) prior to rennet gelation assay and the pH (6.6-6.7) and temperature (30-35°C) at renneting. Such factors would affect the rate of calcium re-solubilisation, and hence, the [Ca^{2+}] at measurement (Chandrapala et al., 2010) and the rennet coagulability (Sandra et al., 2012; Schreiber, 2011).

HHT of milk also affects heat stability. HHT, e.g. at 90°C for 10 min or 110-120°C for 2-3 min, of milk at native milk pH does not alter the overall profile of the HCT/pH curve, but reduces the pH of maximum HCT (HCT_{max}), thereby causing a leftward shift in the HCT/pH curve to lower pH (Sievanen et al., 2008; Singh & Creamer, 1991; Tan-Kintia &

Fox, 1999). Singh and Fox (1985) reported that increasing pH during heat treatment (140°C for 1 min) from 6.7 to 7.3 shifted the HCT_{max} of the resultant milk from 6.5 to 6.4 and increased the HCT at pH values in the range of 6.9-7.1. Despite the importance of HCT in the manufacture of beverages, there have been no further studies on the effect of pH at HHT on the HCT/pH profile when subsequently heating at 140°C. The ethanol stability (ES) test provides a general indication of the stability of the casein to dehydration and aggregation (Horne, 2003). HHT of milk has been found to increase ES in the pH range 6.3-6.7, with the effect becoming more pronounced as the heating time was increased from 10 to 30 min (Horne & Parker, 1981; Mohammed & Fox, 1986). We are unaware of any information on the effect of milk pH at heating on ES.

In many of the previous studies on HHT of milk at different pH, the heat-induced changes in the biochemical/physico-chemical characteristics (e.g., partitioning of minerals and protein between micellar and serum phase, casein micelle size and charge) have not always been evaluated in conjunction with the changes in processing characteristics. Moreover, where heat-induced changes have been measured, these measurements were frequently made at the pH of HHT (e.g. 6.9-7.1), while the processing characteristic (e.g., rennet gelation) was measured at a lower pH (e.g., 6.6-6.7). This confounds the interpretation of the change in processing characteristics with pH at HHT, owing to the effect of pH on casein dissociation, calcium phosphate solubilisation and $[Ca^{2+}]$.

The objective of the current study was to examine the effect of varying the pH of skim milk from 6.6 to 7.5 prior to HHT (95°C) on the physico-chemical and processing characteristics (rennet gelation, heat stability and ethanol stability), and to relate the changes in the latter to the former.

6.2. Materials and methods

6.2.1. Preparation of extra low-heat skim milk powder and skim milk samples

Extra low heat skim milk powder (SMP) was manufactured at Moorepark Technology Ltd (Cork, Ireland). Milk was separated at 55°C to obtain skim milk with ≤0.1% fat (Westfalia Model MM1254 Separator; Westfalia, Germany). Skim milk was evaporated to 45% total solids and spray-dried using centrifugal disc atomization, as described in Chapter 3 and 5. Skim milk samples with 3.3% protein content were prepared by reconstitution of SMP in distilled water at 50°C, storing at 4°C overnight, and then warming at 40°C for 30 min to overcome cold aging (Lin et al., 2016).

6.2.2. pH adjustment and heat treatment of skim milk

Skim milk was adjusted to target pH values of 6.6, 7.2 or 7.5 at 21°C using 3 N HCl or NaOH. Each pH-adjusted skim milk (10 L) was split into 2 portions, one which was left unheated (UH) at 21°C for 1 h, and the other which was subjected to HHT at 95°C for 2 min (HHT), cooled to 15°C using a laboratory scale tubular heat exchanger (MicroThermics®, NC, USA), and equilibrated for 45 min at 21°C. Following equilibration, the pH of both the UH and HHT samples was re-adjusted to 6.6 (similar to that in the milk before heating), equilibrated for 1 h, pH re-adjusted where necessary, and analysed on the same day for preparation of milk serum, rennet gelation characteristics and heat coagulation time. The remainder of each sample was stored at 4°C and analysed within 48 h of heating for gross composition, casein micelle characteristics and ethanol stability; prior to these analyses, the skim milk was warmed to 40°C and held for 30 min to reverse the cold-ageing, and then cooled to 25°C for analysis (Dalgleish & Law, 1988).

The UH and HHT skim milk samples with pH values of 6.6, 7.2 or 7.5 before holding or heating were denoted as UH6.6, UH7.2 and UH7.5, and HHT6.6, HHT7.2 and HHT7.5, respectively.

6.2.3. Preparation of milk ultracentrifugate (serum)

Skim milk was ultracentrifuged at 100,000 *g* for 1 h at 25°C, and the resultant ultracentrifugate (serum) was filtered through glass wool to remove any residual fat (Chapter 2).

6.2.4. Compositional analysis of skim milk and serum

Skim milk samples were assayed for total solids, fat, lactose, casein hydration, casein micelle size (CMS) and zeta potential as described in Chapter 2. Samples of skim milk and serum were analysed for total protein, non-protein nitrogen (NPN) and non-casein nitrogen (at pH 4.6), calcium (Ca), phosphorus (P), and protein profile using reversed-phase high performance liquid chromatography (RP-HPLC), as described in Chapter 2. The analysis scheme used to isolate the different nitrogen (N) /protein fractions of the heated skim milk samples is shown in Figure 6.1a; the measurements performed on the different streams and the parameters derived are shown in Figure 6.1b (Chapter 5). The true protein content of the serum was calculated as the difference between total (crude) protein of serum and NPN (expressed as protein). Total serum casein was calculated as the product of true protein in the serum and casein as a proportion of true protein in the serum, as measured by RP-HPLC.

On pH adjustment of the serum to pH 4.6, serum-soluble casein (κ-, β-, α_s-caseins) and denatured whey protein, assumed to be complexed with κ-casein in the form of serum-soluble aggregates (Mollé et al., 2006), precipitated. The total protein concentration of the pH 4.6-soluble filtrate corresponds to native whey protein and NPN. Hence, the concentration of serum-soluble casein and denatured whey protein/κ-casein aggregates was calculated as the

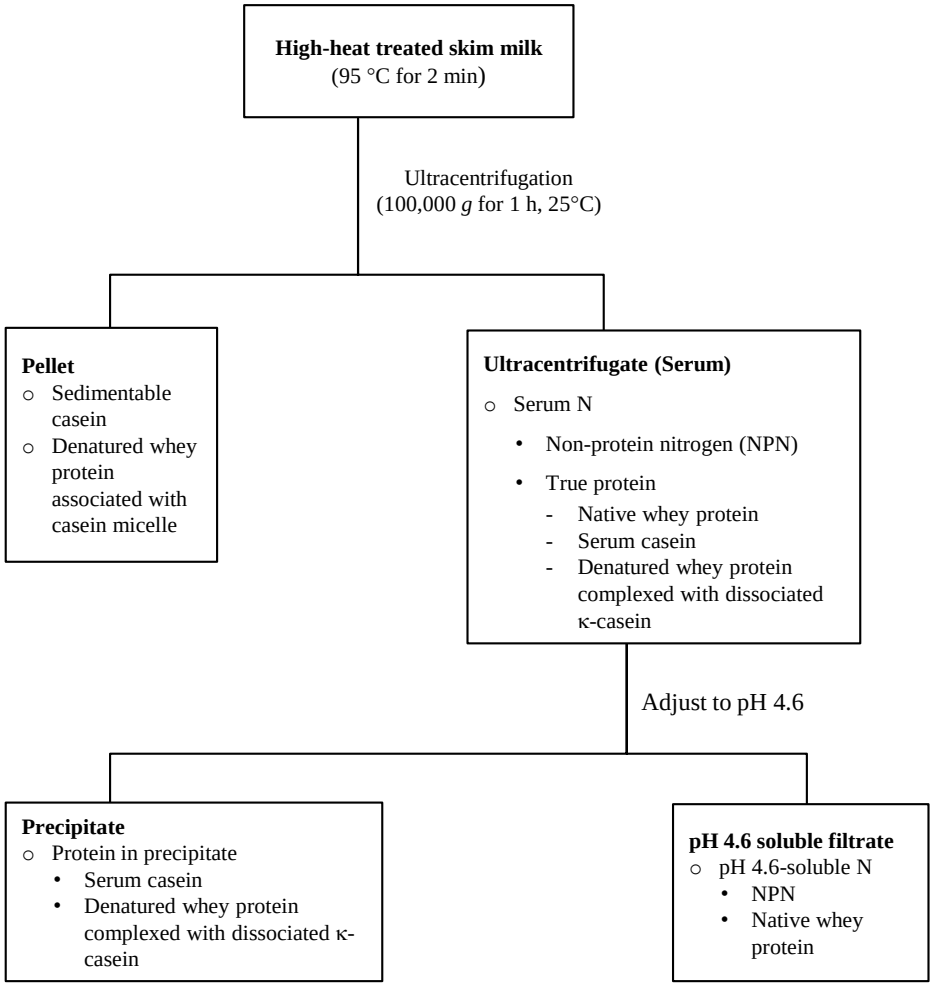
difference in total protein content of the serum and the pH 4.6-soluble filtrate. The difference in concentration between that of the latter and the serum casein corresponds to denatured whey proteins contributing to serum-soluble aggregates. The equations used in the calculation of the different N fractions in the serum phase are below:

(1) True protein in serum (%, w/w) = total protein (%, w/w)-(NPN × 6.38) (%, w/w)

(2) Serum casein (%, w/w) = true protein (%, w/w) × casein (as % of true protein)

(3) Denatured whey protein complexed with dissociated κ-casein (%, w/w) = Total protein (%, w/w)-serum casein (%, w/w) - pH 4.6 soluble protein (%, w/w)

a



b

Stream	Measured	Derived
Skim milk	Total protein, Non-casein N, NPN Protein profile	True protein Whey protein Casein
Serum	Total protein, NCN, NPN Protein profile	True protein (native whey protein, serum casein, serum-soluble denatured whey protein) Serum casein
pH4.6-soluble filtrate	TN	Native whey protein + NPN

Figure 6.1. (a) Flow chart showing the separation of skim milk into different nitrogen (N)/protein fractions, and (b) Analysis undertaken on the different fractions. Abbreviations: N, nitrogen; NPN, non-protein nitrogen; NCN, non-casein nitrogen; TN, total nitrogen.

6.2.5. Processing characteristics of skim milk

Skim milk was adjusted to pH 6.55, heated to 31°C, dosed with chymosin (single strength Chy-Max® plus, 200 IMCU/mL; Chr. Hansen, Hørsholm, Denmark) at a level of 0.17 μl/mL, and monitored for changes in storage modulus, G′, over 1 h, using low-amplitude strain oscillation rheometry, as described in Chapter 2. The following parameters were calculated from resultant G′/time curves: rennet gelation time (RGT), max gel firming rate (GFR_{max}) and storage modulus at 60 min (G'_{60}).

Skim milk samples were adjusted to pH 6.2 to 7.2 and measured for heat coagulation time (HCT) at 140°C, and ethanol stability by blending 1 mL milk with aqueous ethanol solutions (2 mL) with concentration ranging from 32 to 98% (v/v) for 30 s at 21°C (Chapter 2).

6.2.6. Statistical analysis

The data were analysed using a randomized complete block design, which incorporated treatment milk samples (UH and HHT milk samples at different pH before holding or heating) and 2 blocks (separates batches of each treatment milk prepared on 2 separate occasions). Analysis of variance was carried out using a general linear model (GLM) procedure of SAS 9.3 (SAS Institute, 2011) and the effects of treatment and replicate on each response variable were determined. Tukey's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

6.3. Results and discussion

6.3.1. Compositional properties of skim milk samples

HHT or pH adjustment had no effect on gross composition ($P > 0.05$), as evidenced by similar levels of total solids, protein, lactose, Ca and P in the UH and HHT skim milk (Table 6.1). Whey protein denaturation increased significantly with heat treatment at 95°C for 2 min ($P < 0.05$), but was unaffected by the pH before heat treatment. Vasbinder and de Kruif (2003) found that heat-induced denaturation of α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg), as measured by insolubility at pH 4.6, increased slightly (39-42% and 65-71% for α-Lac and β-Lg, respectively) on increasing the pH of the milk from 6.7 to 6.9 before heating at 80°C for 10 min. In contrast, pH had no effect on the extent of whey protein denaturation over the pH range from 6.48 to 6.83 on heating at 80-100°C (Anema & Li, 2003b; Oldfield et al., 2000).

Casein hydration increased with pH in the UH and HHT milk ($P < 0.05$; Table 6.1), an effect most likely associated with the greater negative charge on the casein and hydrogen bonding of water to the casein (de Kruif et al., 2015; Kneifel et al., 1991). Huppertz et al. (2017) also reported an increase in hydration of casein in unheated skim milk as pH increased from 5.7 to 7.2; the authors concluded that ~15% of the total water associated with the casein micelle was non-freezable, or primary hydration, water. HHT resulted in a significant reduction in casein hydration at pH 6.6 and 7.5 ($P < 0.05$). Factors contributing to the reduction in casein hydration are likely to include the precipitation of calcium phosphate, the dissociation of κ-casein at the higher pH values, and the association of denatured hydrophobic whey proteins with the casein micelles (Rüegg & Blanc, 1979).

Table 6.1. Composition of skim milk and serum. [1,2]

	Unheated skim milk			High heat-treated skim milk		
	UH6.6	UH7.2	UH7.5	HHT6.6	HHT7.2	HHT7.5
Skim milk[3]						
Total solids (%, w/w)	8.62^{aA}	8.63^{aA}	8.63^{aA}	8.22^{aA}	8.40^{aA}	8.38^{aA}
Lactose (%, w/w)	4.33^{aA}	4.36^{aA}	4.34^{aA}	4.34^{aA}	4.31^{aA}	4.35^{aA}
Total protein (%, w/w)	3.29^{aA}	3.28^{aA}	3.33^{aA}	3.17^{aA}	3.29^{aA}	3.27^{aA}
Casein (%, w/w)	2.57^{aA}	2.62^{aA}	2.64^{aA}	2.57^{aA}	2.62^{aA}	2.64^{aA}
WP (%, w/w)	0.53^{aA}	0.45^{aA}	0.49^{aA}	0.53^{aA}	0.45^{aA}	0.49^{aA}
Denatured WP (% total WP)	0^{aA}	0^{aA}	0^{aA}	69.6^{aB}	66.6^{aB}	66.6^{aB}
NPN (% TN)	5.87^{aA}	6.57^{aA}	5.95^{aA}	5.90^{aA}	5.66^{aA}	6.39^{aA}
Total Ca (mg/100 g)	110^{aA}	107^{aA}	104^{aA}	107^{aA}	105^{aA}	109^{aA}
Total P (mg/100 g)	93^{aA}	88^{aA}	87^{aA}	88^{aA}	83^{aA}	86^{aA}
Casein hydration (g water/g casein)	3.04^{bA}	3.04^{bA}	3.44^{aA}	2.85^{bB}	3.23^{aA}	3.07^{aB}
Zeta potential (mV)	-22.0^{aA}	-23.1^{aA}	-22.9^{aA}	-22.7^{aA}	-24.5^{aA}	-23.9^{aA}
Skim milk serum						
Protein (%, w/w)	0.95^{bA}	0.99^{bA}	1.16^{aA}	0.54^{bB}	0.88^{aA}	1.07^{aA}
Protein (% milk protein)	28.8^{cA}	30.2^{bA}	34.9^{aA}	16.9^{bB}	26.7^{aA}	32.8^{aA}
Casein (%, w/w)	0.22^{aA}	0.30^{aA}	0.33^{aB}	0.25^{bA}	0.52^{aA}	0.63^{aA}
Casein (% milk casein)	8.5^{aA}	11.5^{aA}	12.6^{aB}	9.8^{bA}	19.9^{aA}	23.9^{aA}
Denatured whey protein associated with κ-casein in serum (% total denatured whey protein)	0^{aA}	0^{aA}	0^{aA}	1.9^{cB}	34.3^{bB}	59.4^{aB}
Ca (mg/100 g)	41.8^{aA}	37.2^{abA}	33.0^{bA}	32.2^{aB}	30.4^{bA}	29.3^{bA}
Ca (% milk Ca)	38.1^{aA}	35.9^{aA}	30.9^{aA}	29.5^{aB}	28.8^{aA}	27.5^{aA}
P (mg/100 g)	39.2^{aA}	33.9^{abA}	31.8^{bA}	33.4^{aB}	31.1^{aA}	30.1^{aA}
P (% milk P)	42.2^{aA}	39.1^{bA}	36.1^{cA}	38.7^{aB}	37.3^{aA}	34.0^{aA}

[1]Skim milk was adjusted to pH 6.6, 7.2 or 7.5, and either held for 1 h at room temperature (unheated: UH) or heated at 95°C for 2 min (HHT), and then readjusted to pH 6.6. Skim milk serum was obtained on ultracentrifugation at 100,000 *g* for 1 h at 25 °C.

[2]Values are the means of duplicate batches for each treatment. Values within a row relating to unheated skim milk (UH6.6, UH7.2 or UH7.5) and not sharing a common lower-case superscript letter differ significantly ($P < 0.05$) for the statistical effect of pH on UH samples. Values within a row relating to high-heat treated skim milk (HHT6.6, HHT7.2 or HHT7.5) and not sharing a common lower-case superscript letter differ significantly ($P < 0.05$) for the statistical effect of pH on HHT samples. Values within a row relating to pH 6.6, 7.2 or 7.5 and not sharing a common upper-case superscript letter differ significantly ($P < 0.05$) for the statistical effect of HHT on skim milk at different pH.

[3]WP, whey protein; NPN, non-protein nitrogen; TN, total nitrogen; Ca, calcium; P, phosphorus.

6.3.2. Casein micelle size (CMS) and zeta potential

HHT at pH 6.6 resulted in an increase in CMS ($P < 0.05$), while heating at pH 7.2 or 7.5 had an opposite effect (Figure 6.2). The increase in CMS on heating at pH 6.6 is consistent with the interaction of denatured whey proteins with κ-casein at the micelle surface (Anema & Li, 2003a, b), as supported by the higher level of sedimentable whey protein in the HHT6.6 milk compared to the UH6.6 milk ($P < 0.05$). The reduction in CMS on HHT at pH 7.2 or 7.5 ($P < 0.05$) is consistent with the higher concentrations of whey protein and κ-casein in the serum of the HHT7.2 and HHT7.5 milk samples compared to the UH7.2 and UH7.5 samples ($P < 0.05$; Table 6.1). In contrast to the results of current study, Ménard et al. (2005) found that increasing the pH incrementally from 6.6 to 8.1 before high heat treatment (90°C for 30 s) resulted in an increase in CMS; the CMS was measured on a suspension prepared by dispersing the casein pellet from high heat-treated milk in native milk ultrafiltrate. The inter-study discrepancy may relate to differences in the composition (Ca, P, κ-casein) of the serum phase in which the heat-treated micelles are located; compared to native milk ultrafiltrate, the serum in HHT milk has lower concentrations of Ca and P, as seen by comparing the serum from UH6.6 skim milk with that from the HHT7.2 or HHT7.5 skim milk.

Increasing the pH of the UH milk before holding led to a slight, but significant, increase in CMS ($P < 0.05$; Figure 6.2). The increase most likely ensues from higher water binding, as evidenced by the increase in casein hydration with pH (Table 1). The trend concurs with that reported by Sinaga et al. (2017), which showed an increase in the CMS of fresh pasteurized skim milk as the pH was increased from 6.6 to 7.5 and held for 24 h at 4°C.

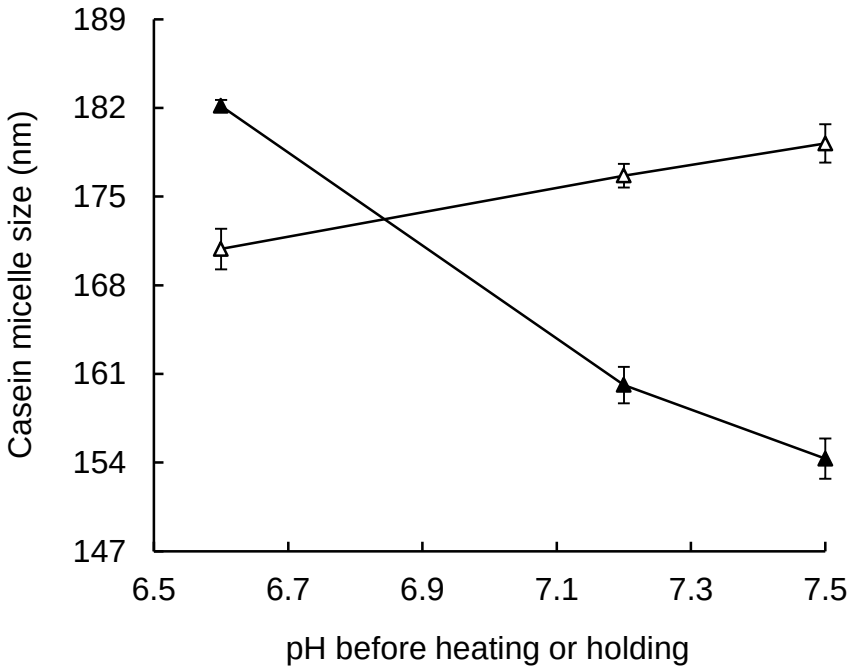


Figure 6.2. Effect of adjusting the pH of skim milk from 6.6 to 7.5 prior to heating (95°C for 2 min, ▲) or holding (unheated skim milk) at room temperature for 1 h (△) on casein micelle size; following heating or holding, the pH of all samples was re-adjusted to pH 6.6. Presented data are the means of duplicate batches; error bars show standard deviation of the mean.

Zeta potential was unaffected by high heat treatment or by increasing pH before heating or holding ($P > 0.05$). This result concurs with the observation of Schmidt and Poll (1986) who found little effect of heating (120°C for 10 min) on the zeta potential of model casein dispersions (casein micelles in simulated milk ultrafiltrate) at pH 6.6-7.0.

6.3.3. Composition of skim milk serum

HHT led to a significant reduction in content of protein in the serum ($P < 0.05$), with the effect becoming less pronounced as the pH prior to HHT was increased from 6.6 to 7.5, as evidenced by smaller differences between the corresponding UH and HHT milk samples as the pH was increased (Table 6.1). The lower concentration of serum protein in the HHT samples is consistent with their

higher level of denatured whey protein (Table 6.1). Similar results were found by Singh and Creamer (1991), who reported that the level of protein in the serum of skim milk decreased from 25 to ~20% of total protein as the heat treatment was increased from 72°C for 15 s to 110°C for 2 min.

Despite the reduction in the concentration of serum protein, the level of serum casein in HHT samples was higher than that of the UH samples, though only significantly at pH 7.5 ($P < 0.05$; Table 6.1). The higher level of serum casein in the HHT skim milk was mainly due to the solubilization of κ-casein, as seen by the higher concentration of κ-casein in the serum of the HHT samples compared to the UH samples at pH 7.2 and 7.5 (Table 6.1, Figure 6.3a,b); the concentrations of serum casein and κ-casein in the UH and HHT skim milk at pH 6.6 were similar. The level of denatured whey protein associated with κ-casein in the serum was higher in the HHT skim milk than in the UH skim milk ($P < 0.05$), to an extent that increased with pH prior to HHT (Table 6.1). In addition to its effect on partitioning of casein and whey protein, HHT led to a significant reduction in the concentrations of serum Ca and P at pH 6.6 ($P < 0.05$) but not at pH 7.2 or 7.5 ($P > 0.05$).

The concentrations of protein, casein and individual α_{s1}-, β- and κ-caseins (as % of corresponding casein in the skim milk) in the serum of the UH and HHT samples increased with pH prior to holding or HHT (Figure 6.3). In contrast to the latter trend, the concentration of serum Ca in the UH and HHT samples decreased on increasing pH before heating or holding ($P < 0.05$). This results for the HHT samples concur with those of Ménard et al. (2005), who showed that κ-casein dissociation in HHT milk increased from <10% to ~60% of total κ-casein as the pH before heating was increased from 6.6 to pH 7.6 or 8.1.

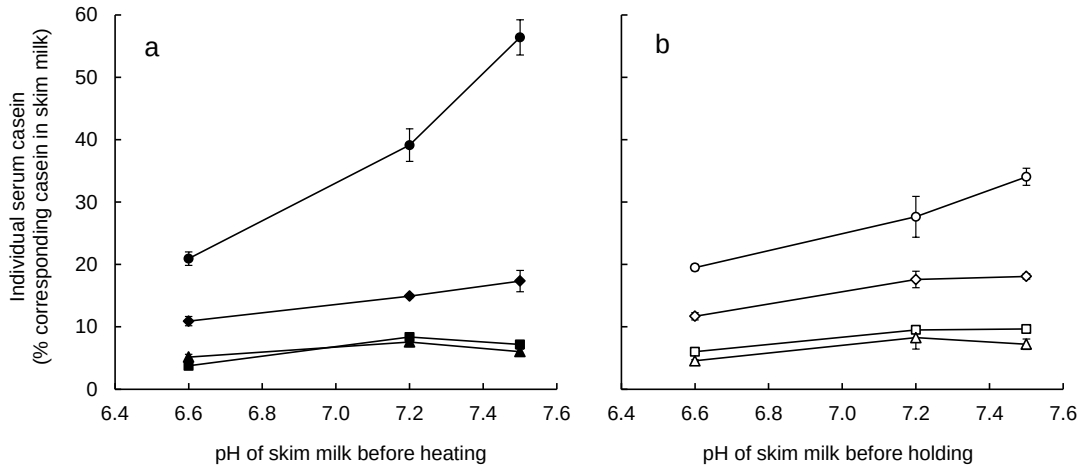


Figure 6.3. Effect of adjusting the pH of skim milk from 6.6 to 7.5 before heating at 95°C for 2 min (a, closed symbols) or holding (unheated skim milk) at room temperature for 1 h (b, open symbols) on the proportion of individual caseins in the serum: α_{s1}- (■,□), α_{s2}- (▲, △), β- (◆,◇) and κ-casein (●,○). Following heating or holding, the pH of all samples was re-adjusted to pH 6.6. Data presented are the means of duplicate batches; error bars represent the standard deviation of the mean.

6.3.4. Rennet gelation

HHT impeded rennet-induced gelation, as indicated by the failure of all HHT milk samples to undergo gelation (Figure 6.S1). The adverse effect of HHT on rennet-induced gelation of milk has been reported extensively in the literature and has been attributed to the reduction in [Ca^{2+}] (Chandrapala et al., 2010; Kethireddipalli et al., 2010; van Hooydonk et al., 1987) and the inhibitory effect of κ-casein-denatured whey protein complexes at the micelle surface on aggregation of the *para*-casein micelles (Ménard et al., 2005; Vasbinder et al., 2003). It has been shown that HHT of milk at pH values higher than native pH (e.g., 7.1) dissociated κ-casein complexes with denatured whey protein to form soluble aggregates, leaving a κ-casein-depleted micelle which is inherently unstable, especially on rennet treatment (Anema et al., 2011; Kethireddipalli et al., 2010; Ménard et al., 2005). However, it has been hypothesized (Anema et al., 2011) that the aggregation and

fusion of the resultant *para*-casein micelles is inhibited by the *para*-κ-casein-whey aggregates.

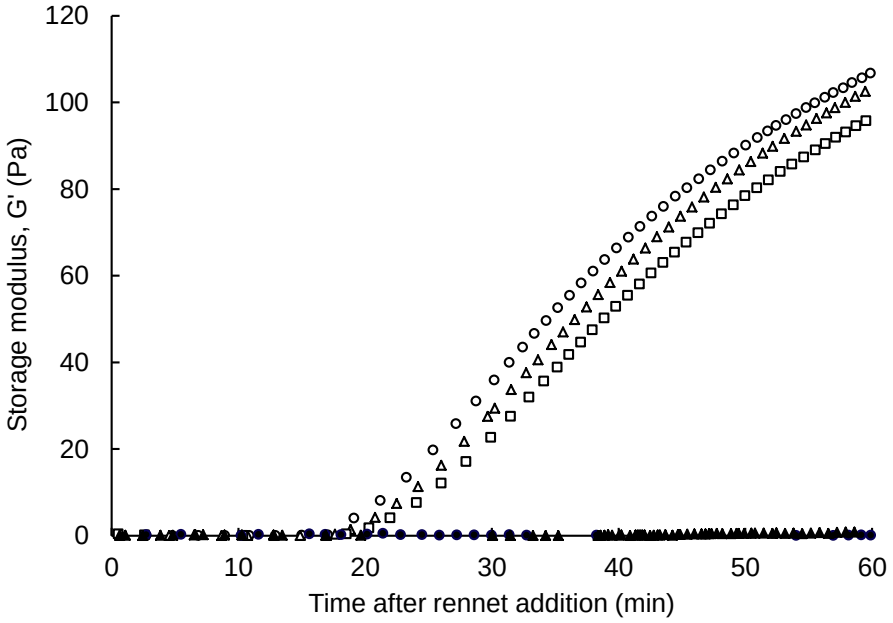


Figure 6.S1. Effect of adjusting the pH of skim milk to 6.6 (●,○), 7.2 (▲, △) and 7.5 (■,□) before heating at 95°C for 2 min (closed symbols) or holding (unheated skim milk) at room temperature for 1 h (open symbols) on the development of storage modulus at pH 6.55. Data presented are for one of the duplicate batches analysed; both batches showed similar trends.

The gelation properties of the UH skim milk samples were slightly, but significantly, impaired on increasing the pH prior to holding ($P < 0.05$), as noted by an increase in rennet gelation time and reductions in G'_{60} and GFR_{max}. A possible explanation for this effect is the reduction in $[Ca^{2+}]$ (Chandrapala et al., 2010), which is supported by the reduction in the concentration of serum Ca (Table 6.1). Furthermore, the increase in serum κ-casein, and hence the reduction in micellar κ-casein may have also contributed; owing to the relatively high hydrophobicity of the *N*-terminal domain of κ-casein (Swaisgood, 2003), casein micelles with a lower content of *para*-κ-casein may have fewer hydrophobic-induced interactions and,

hence, may have a lower susceptibly to aggregation following rennet treatment. In addition, the increase in CMS with pH in the UH milk (Figure 6.2) may have also contributed to the attenuation of gel strength (G'_{60}). Horne et al. (1996) concluded that the gel firming rate is inversely proportional to the cube of the micelle diameter, based on the results of model studies on the rennet-induced gelation of micellar casein dispersions.

6.3.5. Heat coagulation time

HHT shifted the pH of maximum HCT (HCT_{max}) to a lower value (i.e., from 6.8 to 6.7 in HHT7.5 and from 6.7 to 6.6 in HHT6.6 or HHT7.2 samples), resulting in a higher HCT at pH 6.3, 6.4 and 7.2, and lower HCT at pH 6.7 or 6.8 depending on the pH of skim milk before heating (Figure 6.4). This trend concurs with the results of previous studies on the effect of high heat treatment of milk at native pH, i.e., 6.6-6.7 (Sievanen et al., 2008; Singh & Fox, 1985). The reduction in the pH of HCT_{max} in high heat-treated milk is considered to be associated with the increase in the proportion of denatured whey protein interacted with κ-casein at the surface of the casein micelle and the reductions in serum Ca and [Ca^{2+}] (Sievanen et al., 2008; Singh & Creamer, 1991).

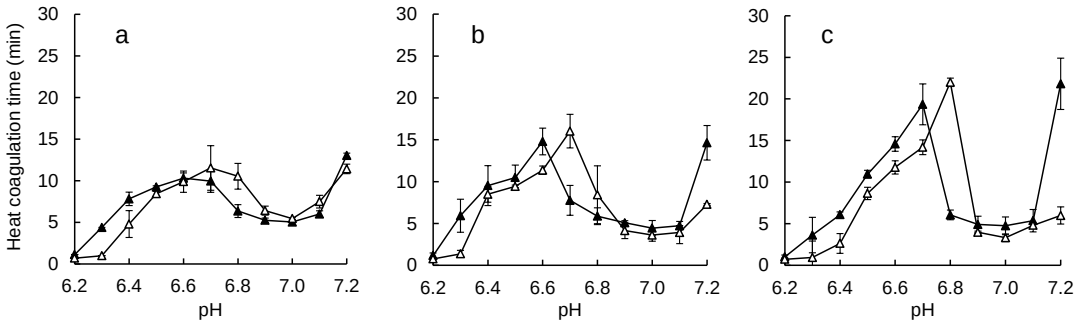


Figure 6.4. Effect of adjusting the pH of skim milk from 6.6 to 7.5 before heating at 95°C for 2 min (▲) or holding (unheated skim milk) at room temperature for 1 h (△) on the heat coagulation time: pH 6.6 (a), 7.2 (b), and 7.5 (c). Following heating or holding, the pH of all samples was re-adjusted to pH 6.6. Data presented are the means of duplicate batches; error bars represent the standard deviation of the mean.

Increasing the pH of the UH or HHT skim milk from 6.6 to 7.5 before holding or heating, respectively, resulted in higher HCT_{max}, higher pH of HCT_{max} (by 0.1 pH unit), and higher HCT at pH 7.2 ($P < 0.05$); nevertheless, the HCT at pH values at pH 6.2-6.5 was scarcely affected ($P > 0.05$). The current results differ somewhat from those of Singh and Fox (1985), who found the pH of HCT_{max} to decrease from 6.5 to 6.4 when the pH of skim milk was increased from 6.7 to 7.1-7.3 prior to HHT (140°C for 1 min). The changes in HCT on alteration of pH before holding or heating (Figure 6.4) coincided with a reduction in serum calcium and an increase in the level of κ-casein dissociation in both UH and HHT skim milk samples. Additionally, increasing the pH of the HHT milk before heating resulted in a significant increase ($P < 0.05$) in the proportion of dissociated κ-casein-whey protein complexes in the serum (Table 6.1). However, the mechanism by which these changes influence heat stability is unclear; perhaps an extended study investigating the compositional and physiochemical changes that occur during HCT measurement would provide greater insight.

6.3.6. Ethanol stability

HHT of skim milk at all pH values (i.e., 6.6, 7.2 and 7.5) significantly enhanced ES in the pH range from 6.2 to 6.6 ($P < 0.05$), but had no effect at pH 6.8-7.0 (Figure 6.5). The positive effect of HHT on the ES of skim milk in the mid-pH range has been previously reported and attributed to a reduction in $[Ca^{2+}]$ (Horne & Parker, 1981; Mohammed & Fox; 1986). Apart from giving a reduction in the ES at pH 6.6 of the UH sample ($P < 0.05$), increasing the pH before holding or heating of the UH and HHT samples, respectively, had no effect on the ES at pH 6.2-7.0. It might be expected that the higher degree of κ-casein dissociation and lower CMS in the HHT samples heated at 7.2 or 7.5 (Figure 6.3) would render the casein micelle

more susceptible to ethanol-induced flocculation, and thereby give a more compact floc and lower ES. The current results suggest that the effect of κ-casein dissociation and CMS may have been off-set by a lower concentration of serum Ca (Table 6.1), and hence [Ca^{2+}], in the latter samples.

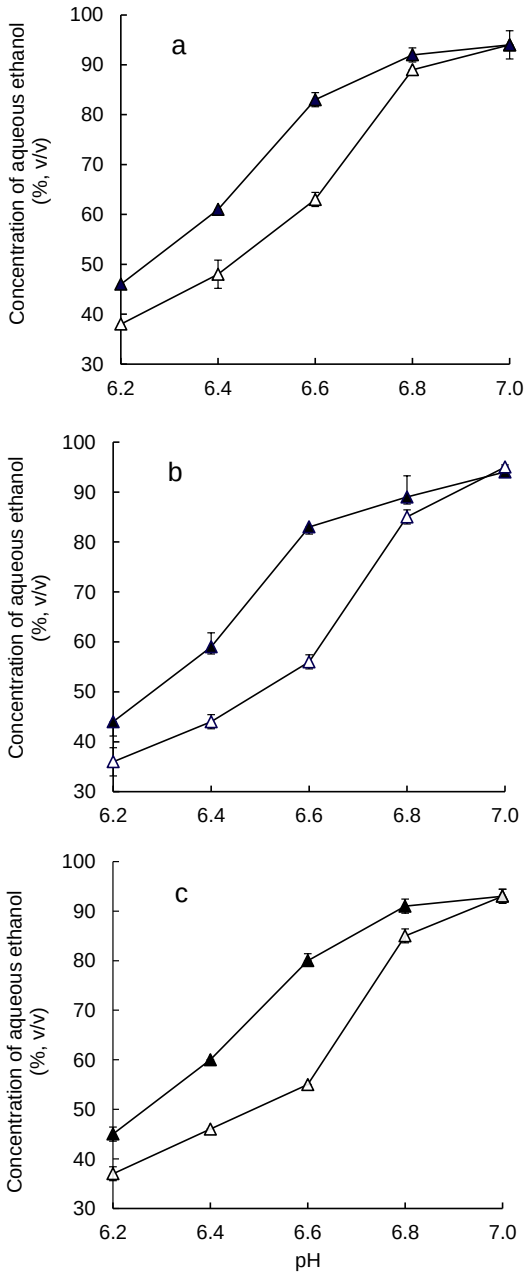


Figure 6.5. Effect of adjusting the pH of skim milk from 6.6 to 7.5 before heating at 95°C for 2 min (▲) or holding (unheated skim milk) at room temperature for 1 h (△) on the ethanol stability: pH 6.6 (a), 7.2 (b), and 7.5 (c). Data presented are the means of duplicate batches; error bars represent the standard deviation of the mean.

6.5. Conclusion

HHT of skim milk impaired rennet gelation, and altered the ES and HCT to an extent dependent on the pH at heating. These effects were associated with increases in whey protein denaturation and κ-casein dissociation, and, a reduction in the concentration of serum-soluble Ca. Increasing the pH of the skim milk prior to HHT led to increases κ-casein dissociation and serum-soluble denatured whey protein, and reductions in serum Ca and CMS. The fact that ES and HCT of skim milk can be manipulated by altering the pH before heating is of relevance to the users of skim milk powder in applications such as recombined milks, heated milk-based beverages, and alcoholic milk-based beverages. Moreover, when used in conjunction with novel separation techniques, it may provide an approach for development of new ingredients (e.g., κ-casein, β-Lactoglobulin enriched powders; κ-casein depleted micelles).

6.6. Acknowledgements

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Chapter 7

Effects of milk heat treatment and solvent composition on physico-chemical and selected functional characteristics of milk protein concentrate

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Abstract

Milk protein concentrate powders (MPC, ~81% protein) were made from skimmed milk which was heat-treated at 72°C for 15 s (LHMPC) or 85°C for 30 s (MHMPC). The MPC powder was manufactured by ultrafiltration (UF) and diafiltration (DF) of skim milk at 50°C followed by spray drying. MPC dispersions (4.02% true protein) were prepared by reconstituting the LHMPC and MHMPC powders in distilled water ($LHMPC_w$ and $MHMPC_w$, respectively) or milk permeate ($LHMPC_p$ or $MHMPC_p$, respectively). Increasing milk heat treatment increased the level of whey protein denaturation (from ~5 to 47% of total whey protein) and reduced the concentrations of serum protein, serum calcium and ionic calcium. These changes were paralleled by impaired rennet-induced coagulability of the $MHMPC_w$ and $MHMPC_p$ dispersions, and a reduction in the pH of maximum heat stability of $MHMPC_p$ from pH 6.9 to 6.8. For both the LHMPC and MHMPC dispersions, the use of permeate instead of water enhanced ethanol stability at pH 6.6–7.0, impaired rennet gelation, and changed the heat coagulation time/pH profile from type A to type B. Increasing the severity of milk heat treatment during MPC manufacture and the use of permeate instead of water led to significant reductions in the viscosity of stirred yoghurt prepared by starter-induced acidification of the MPC dispersions. The current study clearly highlights how the functionality of protein dispersions prepared by reconstitution of high protein MPC powders may be modulated by the heat treatment of the skim milk during manufacture of the MPC and the composition of the solvent used for reconstitution.

Key words: milk protein concentrate, milk heat treatment, solvent composition, functionality

7.1. Introduction

Developments in membrane filtration of milk since the 1970s have led to the availability of a range high protein powders including milk protein concentrates (MPCs), micellar caseins, whey protein concentrates/isolates, and α-lactalbumin (α-La). MPCs with high protein content (e.g., ≥80%, MPC 80) are prepared by concentration of milk protein (casein and whey protein) using ultrafiltration (UF) and diafiltration (DF) of the resultant retentate to dilute out most of the milk serum and its solids components, including lactose, soluble salts and non-protein nitrogen (NPN). Huppertz and Gazi (2015) reported that the level of denaturation of β-lactoglobulin in commercial MPC powders varies from ~20 to 80% of total, indicating that the milk heat treatment applied during MPC manufacture varies extensively.

MPCs are used extensively in food manufacture/formulation, with applications including dairy-based beverages, yoghurt, fresh-cheese products, recombined milk cheeses, ice-cream, coffee whitener, high protein bars, and alcoholic dairy beverages). During food formulation, MPC is exposed to environments differing substantially in total solids content, the types and levels of different ingredients, the composition of the solvent phase (e.g., ionic strength, pH, sugar content) and processing conditions (e.g., heat, acidification, rennet gelation, addition of ethanol). Nevertheless, MPC must provide the requisite functionalities or combinations thereof, including emulsification, gelation, foaming, heat stability and/or nutritive value (Patel & Patel, 2014; Ikeda, 2015). High protein MPC powders are more functional than other ingredients, such as skim milk powder or whey protein concentrates, in many applications owing to the combined functionalities of

both casein and whey protein, their neutral flavour (for example compared to sodium caseinate), and low lactose content (< 3%). Lactose is a non-functional ingredient (i.e., inert carbohydrate filler) in many formulations and high levels increase formulation cost, the risk of crystal formation in products such as ice cream, and browning in products subjected to high temperature conditions during manufacture (e.g., ultra-high heat treated products) or food service (e.g., formulated foods that are baked/grilled).

A number of recent studies have reported the effects of manufacturing conditions on the functionality of high protein MPC, including heat treatment of the skim milk prior to UF (Crowley et al., 2015; Gazi & Huppertz, 2015), alteration of calcium (Ca) content by pre-acidification of the skim milk prior to UF and/or DF (Luo et al., 2016; Eshpari et al., 2017), lowering the temperature of the milk during UF (Luo et al., 2015), addition of NaCl (Mao et al., 2012) or calcium-chelating salts to the skim milk (Ramchandran et al., 2017) prior to UF or the retentate prior to DF (Bhaskar et al., 2001; Guinee et al., 2009), or high-pressure treatment of the skim milk prior to UF (Udabage et al., 2012). Increasing severity of milk heat treatment from 72°C for 15 s to 95°C for 45 s led to denaturation of 65% of total β-lactoglobulin (β-Lg) and 25% of total α-La (Gazi & Huppertz, 2015), but had little impact on the heat coagulation time (HCT) of aqueous dispersions of the MPC (8.5% protein) at 120°C in the pH range 6.3–7.1 (Crowley et al., 2015).

The effect of solvent quality on the functionality of dispersions prepared from high protein MPC has also been investigated. Crowley et al. (2014) evaluated the effect of substituting water with simulated milk

ultrafiltrate (SMUF) or SMUF with lactose (4.6%) and urea (30 mg/ 100 g) on the HCT of protein dispersions (3.5%) prepared from low-heat MPC with 80% protein (w/w; MPC80). The HCT of a water-based dispersion of MPC80 (3.5% protein) at 140°C remained very low (< 2 min) at pH 6.3–6.9 and then increased as the pH was further increased to 7.2. The use of SMUF or SMUF-lactose instead of water introduced a maximum HCT (HCT_{max}) at pH 6.7–6.8. However, cold dialysis of the water-based dispersion against reconstituted skim milk resulted in a type A HCT *vs.* pH profile with a HCT_{max} at 6.0 and minimum HCT (HCT_{min}) at pH 7.1. There are two types of HCT *vs.* pH profile for bovine milk; type A, which is the most common, is characterised by a maximum and minimum HCT at pH 6.6-6.7 and 6.8-7.0, respectively, and type B, for which HCT increases progressively with pH increases in the range 6.2 to 7.2 (O'Connell and Fox, 2003). Eshpari et al. (2015, 2017) altered the solvent composition of protein dispersions (3.2% protein) prepared from standard- or reduced-Ca MPC 80 by overnight dialysis against skim milk at 4°C. The pH of the non-dialysed standard-Ca and reduced-Ca samples was 7.1 and 6.68, respectively, while that of the corresponding dialysed standard-Ca and reduced-Ca samples was 6.65 and 6.65, respectively. Dialysis increased the concentrations of non-sedimentable protein and Ca of both the standard-Ca and reduced-Ca dispersions, the HCT of the reduced-Ca dispersion, and the storage modulus (G') of the rennet-treated standard-Ca dispersion. Meletharayil et al. (2016) studied the effects of increasing lactose content (~0.3, 5.6 and 11.2%, w/w), on the glucono-δ-lactone (GDL)-induced gelation of 4% protein dispersions prepared from low-heat MPC80. Increasing lactose content from 0.3 to 11.2% coincided

with increases in the pH at onset of gelation (from ~ pH 5.3 to 5.6) and G' at pH 4.6 (from ~340 to 460 Pa), and a reduction in the level of expressible serum (whey) on centrifugation at 3000 × *g* (from 67 to 36 g/100 g).

To our knowledge, there is no comprehensive study on the combined effects of milk heat treatment and solvent composition on the functionality of MPC dispersions. The objectives of the current study were to investigate the effects of milk heat treatment (72°C for 15 s or 85°C for 30 s) during the manufacture of MPC powder, and the solvent (water or milk permeate) used for reconstitution of the MPC powder on the composition, physicochemical, and key functional characteristics of the resultant MPC protein dispersions (4% true protein). Commercially, water and milk permeate are commonly used solvents in formulated food products.

7.2. Materials and methods

7.2.1. Manufacture of low-heat and medium-heat MPC

MPC was produced in the Bio Functional Food Engineering pilot-plant unit of Moorepark Technology Limited (Teagasc, Moorepark, Fermoy, Co. Cork). Milk was separated at 55°C (Westfalia Model MM1254 Separator; Westfalia, Germany). Skim milk (~800 L) was split into two portions (~400 L), one of which was used for the manufacture of low-heat MPC (LHMPC), and the other for medium-heat MPC (MHMPC). Milk was pasteurized at 72°C × 15 s using a plate heat-exchanger (APV Pasilac SSP pilot plant, APV DK 8600, Silkeborg, Denmark) for LHMPC or at 85°C for 30 s using a pilot-scale tubular heat-exchanger (MicroThermics, NC, USA) for MHMPC.

The pasteurized skim milk was ultrafiltered at 50°C (10 kDa; total membrane area: 27 m^2; ST28 3838 UF membrane, Synder® Filtration, California, USA) to 21% total solids (TS). The resultant retentate was diluted with deionized-water (50°C) at a retentate:water weight ratio of 1:1, diafiltered to 21% TS using UF at 50°C, and spray-dried (Anhydro Spray Dryer, SPX Flow Technology Danmark A/S, Soeborg, Denmark) using nozzle atomization at inlet and outlet air temperatures of 180 and 85°C, respectively. The LHMPC and MHMPC powders (~4 kg of each type) were packed in silver aluminium bags and stored at 15°C until used for analysis.

Both LHMPC and MHMPC were each produced on two separate occasions (trials), with both powder types being produced from the same milk on each occasion.

7.2.2. Milk permeate

During the preparation of MPC, a portion (10 L) of permeate obtained during ultrafiltration of the pasteurized skim milk (72°C × 15 s) was collected. The sample was divided into 250-mL quantities, each of which was rapidly frozen in liquid nitrogen and then placed in a freezer at -20°C until required.

7.2.3. Preparation of protein dispersions from MPC powders

Protein dispersions with 4.02% true protein (w/w) were prepared by dispersing the MPC powder in distilled water (MPC_w) or milk permeate (MPC_p) at 50°C while continually stirring at 500 rpm (IKA® RT10 Magnetic Stirrer, IKA-Werke GmbH & Co. KG, Staufen, Baden-Württemberg, Germany) for ~2 h and holding/stirring overnight at 4°C to ensure protein

hydration. Prior to analysis, the MPC dispersions were warmed to 40°C and held for 30 min to reverse cold-aging, and then cooled to 25°C for analysis.

To eliminate the effect of the difference in pH between the MPC_w (~7.0) and MPC_p (~6.65) on the serum composition, particle size and zeta potential of the water- and permeate-based protein dispersions, the pH of the sub-samples of the water-based dispersions were adjusted to pH 6.65 at room temperature. The protein dispersions prepared from the LHMPC powder in distilled water, water followed by pH adjustment to 6.65, or milk permeate are denoted as $LHMPC_w$, $LHMPC_{w\text{-}pHa}$ and $LHMPC_p$, respectively; the corresponding dispersions prepared from MHMPC powder are denoted as $MHMPC_w$, $MHMPC_{w\text{-}pHa}$ and $MHMPC_p$, respectively.

7.2.4. Solubility of milk protein dispersions

The solubility of MPC dispersions after preparation and after overnight hydration at 4°C, while stirring, was determined by measuring the percentage of total solids that remained non-sedimentable on centrifugation at 700 × g for 10 min at 24°C, using the method described by Carr (1999). TS were measured using the CEM SMART Trac II (CEM, North Carolina, USA). Solubility (S) is expressed as % solubility, defined as % TS in supernatant as a percentage of TS in the original dispersion.

The solubility was also determined indirectly, by measuring the insolubility index using a modification of the International Dairy Federation (IDF) standard method for dried milk and dried milk products (IDF, 1989). The modifications involved using a weight (~5 g) sufficient to give a true protein content of 4.02% (w/w), and permeate instead of water as solvent for

the preparation of the $LHMPC_p$ and $MHMPC_p$ dispersions. The dispersions (24°C) were centrifuged at 900 rpm (Funke Gerber, type SuperVario-N; Funke-Dr.N.Gerber Labortechnik GmbH, Berlin, Germany) and the volume of sediment (mL) measured visually.

7.2.5. Ultracentrifugation of milk protein dispersions

Protein dispersions were ultracentrifuged at 100,000 × *g* at 25°C for 1 h to determine the proportions of sedimentable and non-sedimentable proteins and minerals. The resultant supernatant was filtered through glass wool to ensure removal of any residual fat. The sediment layer (pellet) was lyophilised at -46°C (FreeZone Freeze Dry Systems, Labconco, Kansas City, USA) under vacuum ($\leq 130 \times 10^{-3}$ mBar).

7.2.6. Compositional analysis of milk permeate, MPC powder, protein dispersion, supernatant and UF permeate

MPC powders were characterized for gross composition including fat and TS using the CEM SMART Trac II, lactose using a Megazyme Lactose/Galactose Assay Kit (Megazyme International Ireland, Bray Business Park, Bray, Co. Wicklow, Ireland), and total protein (TP), non-casein nitrogen (NCN), NPN, Ca and P by International Dairy Federation, IDF, standard methods, as described by Lin et al. (2016). The protein dispersion, supernatant obtained on ultracentrifugation, and UF permeate were analysed for TP, NCN, NPN, Ca and P using IDF standard methods and individual caseins by reversed phase high pressure liquid chromatography as described by Lin et al. (2016). Additionally, the protein dispersions were assayed for fat and TS using the CEM SMART Trac II, and lactose by the FOSS MilkoScan FT+ (Foss Electric A/S, Hillerød, Denmark). The concentration of ionic calcium ($[Ca^{2+}]$) was measured at room temperature using the sensION+ 9660C Calcium

Combination Ion Selective Electrode (Hach Lange, Barcelona, Spain), where calibration was performed using $CaCl_2$ solutions (0.0-10.0 mM), as described in Chapter 3.

7.2.7. Physico-chemical characteristics of protein dispersions

CMS, expressed as z-average (nm), and zeta potential of the protein dispersions were determined using a Malvern Zetasizer Nanoseries Nano-ZS (Malvern Instruments Ltd, Malvern, UK), as described in Chapter 2. Casein hydration was measured by lyophilisation of the pellet obtained on ultracentrifugation, and expressed as g water/g sedimented casein (Chapter 2)

7.2.8. Rennet gelation

Protein dispersions were adjusted to pH 6.55 at 21°C and equilibrated for 15 min. Chymosin (single strength Chy-Max® plus, 200 IMCU/mL; Chr. Hansen, Hørsholm, Denmark) was diluted 20-fold in distilled water and added to give 1.03 IMCU/g protein. Gel formation was monitored by measuring the change in storage modulus, G', over time using low-amplitude strain oscillation rheometry (LASOR; Carri-Med, type CSL^2_{500}, TA instruments, New Castle, DE, USA). The following parameters were calculated from the resulting G' *vs.* time curves as described by Lin et al. (2016): rennet gelation time (RGT), maximum gel firming rate (GFR_{max}) and storage modulus at 60 min (G'_{60}).

7.2.9. Heat coagulation time and ethanol stability

The pH of protein dispersions was adjusted to values in the range 6.2 to 7.2 at 21°C by incremental addition of 0.1 N HCl or NaOH. The HCT of pH-adjusted protein dispersions was measured in a thermostatically controlled oil bath (Hettich

Elbanton Special Product, Hettich Benelux Laborator Equipment, Geldermalsenat, Netherlands) at 140°C, as described previously (Lin et al., 2016).

For the measurement of ES, the protein dispersions were adjusted to pH values ranging from 6.2 to 7.0 at 21°C. The pH-adjusted samples were blended with aqueous ethanol solutions ranging in concentration from 30 to 98% (v/v) at a volume ratio of 1:2.4 (protein dispersion: ethanol solution), and the mixture was agitated for 30 s (Whirlimixer™, Fisons, Holmes Chapel, UK). ES was recorded as the minimum concentration of aqueous ethanol solution required to induce flocculation.

7.2.10. Model stirred skimmed yoghurt preparation and gel formation

Protein dispersions (1 L) with 5.0% true protein in water with added α-lactose monohydrate powder or in milk permeate, were prepared as described above; α-lactose monohydrate powder (> 99.0% lactose; Arla Foods Ingredients, Sønderhøj, Denmark) was added to the water to give a total lactose level of 4.8% (w/w). The dispersions were heated to 85°C, held for 20 min while stirring continuously at 200 rpm (Model RW16; IKA-Werke GmbH, Staufen im Breisgau, Germany), cooled to 42°C in an ice-water bath, inoculated with direct-vat starter cultures YC380 and CH1 YoFlex® (consisting of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* at weight ratio of 1:3; Chr. Hansen Ireland Ltd, Rohan Industrial Estate, Little Island, Co. Cork, Ireland) and incubated at 42°C (Heratherm™ Advance Protocol Microbiological Incubators, Thermo Scientific™, Massachusetts, USA) until the pH reached 4.6. Subsamples (~20 mL) were withdrawn periodically during incubation, cooled to room temperature, and monitored for pH. The gelled dispersion was cooled to < 8°C in ice-water while stirring at 70 rpm (Model RW16; IKA-

Werke GmbH, Staufen im Breisgau, Germany), and stored at 4°C for 36 h prior to analysis.

Immediately, after starter culture inoculation, a well-mixed subsample (10 mL) of the dispersion was withdrawn and monitored for changes in loss modulus (G″), G' and loss tangent (tanδ; tanδ = G″/G') over a 9 h period at 42°C using LASOR, as described for rennet gelation. Moisture evaporation from the sample during measurement was prevented by placing a thin layer of tetradecane (Sigma-Aldrich, Massachusetts, United States) on the surface of the sample protein dispersion and covering the sample with an evaporation blocker.

7.2.11. Water holding capacity (WHC) of yoghurt

Immediately after cooling to 8°C, sub-samples (4) of each yoghurt were poured into 50 mL stoppered centrifuge tubes, held at 4°C for 36 h, and centrifuged at 300 or 2500 × *g* at 8°C for 30 min; the expressed serum was decanted and weighed. The WHC was calculated as the total serum less the serum expressed on centrifugation per 100 g yoghurt.

7.2.12. Rheological properties of stirred yoghurt

Yoghurt was stirred at 70 rpm for 1 min at room temperature (Model RW16; IKA-Werke GmbH, Staufen im Breisgau, Germany) to ensure homogeneity. A sub-sample (10 g) was placed in the measuring cell of a controlled-stress rheometer (Carri-Med, type CSL^2_{500}, TA Instruments, New Castle, DE, USA). The cell consisted of two coaxial cylinders, an outer cup (internal diameter 27.5 mm) and an inner bob (diameter 25 mm). Following equilibration at 8°C for 5 min, the sample was then subjected to a shear rate, $\dot{\boldsymbol{\gamma}}$, sweep, whereby $\dot{\gamma}$ was increased from 10 to 120 s^{-1}. Shear stress ($\boldsymbol{\sigma}$; Pa) and viscosity ($\boldsymbol{\eta}$; Pa.s) were measured as a function of $\dot{\gamma}$.

The resultant $\dot{\gamma}$ *vs.* σ data were fitted to Herschel-Bulkley model using TA data analysis software (TA Rheology Advance Data Analysis, Version V5.7.0, New Castle, DE, USA):

$$\sigma = \sigma_o + K \dot{\gamma}^{n},$$

where σ_o, K, and n represent yield stress (Pa), consistency coefficient (Pa.s) and flow behaviour index (n), respectively (Ramaswamy and Basak, 1991).

7.2.13. Statistical analysis

The data were analysed using a randomized complete block design, which incorporated the protein dispersions ($LHMPC_W$, $MHMPC_W$, $LHMPC_P$ and $MHMPC_P$) and 2 replicate blocks (samples prepared from the 2 separate trials of MPC made on different days). Analysis of variance (**ANOVA**) was carried out using a general linear model (**GLM**) procedure of SAS 9.3 (SAS Institute, 2011) and the effects of heat treatment and reconstitution medium on each response variable were determined. Tukey's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

7.3. Results and discussion

7.3.1. Composition of MPC powders and milk permeate

The compositions of heat-treated skim milk, MPC powders (LHMPC and MHMPC) and milk permeate (from low heat skim milk) are shown in Table 7.1. Increasing the severity of heat treatment of the skim milk from 72 to 85°C resulted in an increase in the level of whey protein denaturation from 5.6 to 47% of total whey

protein; otherwise, the gross composition of the LH and MH skim milk samples was similar.

The composition of the LHMPC and HHMPC powders is similar to that reported for high-protein commercial (Patel & Patel, 2014) and experimental MPC powders (Martin et al., 2010; Crowley et al., 2015). There was no significant difference between the levels of TS, TP, lactose, fat, Ca or P between the LHMPC and MHMPC powders.

Table 7.1. Composition of unheated skim milk, heat-treated skim milk (LHSM, MHSM), milk protein concentrate powders (LHMPC, MHMPC) and milk permeate.[1,2,3]

	Effect of heat treatment on skim milk			Effect of heat treatment on MPC powders		Milk permeate
	Skim milk (unheated)	LHSM	MHSM	LHMPC powder	MHMPC powder	
Total solids (%, w/w)	89.9[a]	90.0[a]	90.3[a]	4.60[a]	4.56[a]	5.04
Protein (% w/w)	4.23[a]	4.24[a]	4.23[a]	81.34[a]	81.54[a]	0.22
Casein (% w/w)	3.47[a]	3.47[a]	3.47[a]	67.81[a]	67.82[a]	0.00
WP (%, w/w)	0.56[a]	0.56[a]	0.57[a]	12.97[a]	12.97[a]	0.01
WP denaturation (% total WP)	0.0[c]	5.7[b]	46.9[a]	5.7[b]	46.9[a]	n/a
NPN, expressed as protein (% w/w)	0.23[a]	0.23[a]	0.23[a]	0.58[a]	0.54[a]	0.21
Lactose (% w/w)	4.84[a]	4.86[a]	4.80[a]	2.66[b]	2.42[b]	4.79
Fat (%, w/w)	0.06[a]	0.05[a]	0.05[a]	1.40[a]	1.42[a]	0.00
pH	6.67[a]	6.66[a]	6.66[a]	-	-	6.58
Na (mg/100 g)	-	-	-	63[a]	75[a]	52
K (mg/100 g)	-	-	-	222[a]	224[a]	165
Ca (mg/100 g)	148[a]	147[a]	147[a]	2409[a]	2441[a]	28
P (mg/100 g)	100[a]	99[a]	99[a]	1360[a]	1348[a]	33
Mg (mg/100 g)	-	-	-	108[a]	109[a]	10

[1]Sample codes: LHSM, low-heat-treated skim milk (72°C for 15 s); MHSM: medium-heat-treated skim milk (85°C for 30 s); LHMPC and MHMPC denoted milk protein concentrate powders from LHSM and MHSM, respectively.

[2]Values in a row relating to effect of heat treatment of skim milk and not sharing a superscripted letter differ significantly ($P < 0.05$), and values in a row relating to effect of heat treatment on milk protein concentrate (MPC) powder and not sharing a superscripted letter differ significantly ($P < 0.05$).

[3]WP, whey protein; NPN, non-protein nitrogen; TN, total nitrogen; Ca, calcium; P, phosphorus; n/a, not applicable; -, not determined.

7.3.2. Solubility of MPC powders

The solubility of the MPC powders in water or permeate, following overnight holding at 4°C and heating of the dispersions at 40°C for 30 min to reverse cold ageing, varied from 95.5 to 96.8%; it was unaffected by heat treatment of the milk used in MPC manufacture or the solvent used for reconstitution of the MPC (Table 7.2). The solubility values are comparable to those (> 95%) reported by Gazi and Huppertz (2015) for MPC powders with protein levels of 35-85%, in water.

The insolubility index of MPCs in water or permeate decreased from ~ 2.75–3.75 mL following dispersion to <0.18 ml sediment (Table 7.2) after overnight holding at 4°C, indicating the beneficial effect of cold storage on protein hydration. This observation concurs with the findings of Ferrer et al. (2008) who found that overnight holding of protein dispersions from MPC powders with 56–90% protein was accompanied by a reduction in particle size where the dispersions were not subject to high shear (homogenization) during preparation.

Table 7.2. Compositional and physico-chemical characteristics of milk protein dispersions.[1,2,3]

	Water-based protein dispersion		Water-based protein dispersion, pH-adjusted to 6.65		Permeate-based protein dispersion	
	$LHMPC_w$	$MHMPC_w$	$LHMPC_{w\text{-}pHa}$	$MHMPC_{w\text{-}pHa}$	$LHMPC_p$	$MHMPC_p$
Protein dispersion						
Total solids (%, w/w)	4.9^{aB}	4.8^{aB}	-	-	10.3^{aA}	10.1^{aA}
Lactose (%, w/w)	0.1^{aB}	0.1^{aB}	-	-	4.8^{aA}	4.7^{aA}
Total protein (%, w/w)	4.06^{aB}	4.02^{aB}	-	-	4.26^{aA}	4.22^{aA}
Casein (%, w/w)	3.4^{aA}	3.4^{aA}	-	-	3.4^{aA}	3.4^{aA}
WP (%, w/w)	0.63^{aA}	0.61^{aA}	-	-	0.69^{aA}	0.66^{aA}
NPN (% TN)	0.67^{aB}	0.62^{aB}	-	-	5.01^{aA}	5.01^{aA}
Total Ca (mg/100g)	119^{aB}	121^{aB}	-	-	149^{aA}	147^{aA}
Ca (mg/g casein)	35.4^{aB}	35.8^{aB}	-	-	44.4^{aA}	43.5^{aA}
Total P (mg/100 g)	68^{aB}	67^{aB}	-	-	103^{aA}	97^{aA}
Ionic Ca ($[Ca^{2+}]$,	4.01^{aC}	3.64^{aC}	7.77^{aA}	7.26^{bA}	6.48^{aB}	5.85^{bB}
pH	6.98^{aA}	6.99^{aA}	6.65^{aB}	6.65^{aB}	6.64^{aB}	6.65^{aB}
Solubility						
- Solubility (%)	95.4^{aA}	96.8^{aA}	-	-	96.6^{aA}	96.5^{aA}
- Sediment volume before hydration (mL)	2.50^{aA}	3.75^{aA}	-	-	2.75^{aA}	3.75^{aA}
- Sediment volume after hydration (mL)	0.13^{aA}	0.06^{aA}	-	-	0.17^{aA}	0.12^{aA}
Casein hydration (g water/g casein)	3.22^{aA}	3.21^{aA}	3.25^{aA}	3.21^{aA}	3.26^{aA}	3.26^{aA}
Zeta potential (mV)	-28.0^{aA}	-28.0^{aA}	-24.2^{aB}	-23.1^{aB}	-19.9^{aC}	-20.3^{aC}
Particle size (nm)	198^{bA}	219^{aA}	190^{bB}	209^{aB}	158^{bC}	168^{aC}

[1]Sample codes: Low-heat and medium-heat milk protein concentrate powders (LHMPC, MHMPC), manufactured from low heat-treated (72°C for 15 s) or medium heat-treated (85°C for 30 s) skim milk, were dispersed in water ($LHMPC_w$, $MHMPC_w$) or milk permeate ($LHMPC_p$, $MHMPC_p$). Sub-samples of $LHMPC_w$ and $MHMPC_w$ were pH adjusted to pH 6.65 and denoted as $LHMPC_{w\text{-}pHa}$ and $MHMPC_{w\text{-}pHa}$, respectively.

[2]Values in a row relating to effect of milk heat treatment ($LHMPC_w$ and $MHMPC_w$, $LHMPC_{w\text{-}pHa}$ and $MHMPC_{w\text{-}pHa}$, and $LHMPC_p$ and $MHMPC_p$) and not sharing a common superscripted lower-case letter differ significantly ($P < 0.05$); values in a row relating to the effect of solvent ($LHMPC_w$, $LHMPC_{w\text{-}pHa}$ and $LHMPC_p$; and $MHMPC_w$, $MHMPC_{w\text{-}pHa}$ and $MHMPC_p$) and not sharing a common superscripted upper-case letter differ significantly ($P < 0.05$).

[3]WP, whey protein; NPN, non-protein nitrogen; TN, total nitrogen; Ca, calcium; P, phosphorus; -, denotes not measured.

Table 7.3. Compositional and physico-chemical characteristics of milk protein dispersions, and the serum obtained on ultracentrifugation.[1,2,3,4]

	Water-based protein dispersion		Water-based protein dispersion, pH-adjusted to 6.65		Permeate-based protein dispersion	
	$LHMPC_w$	$MHMPC_w$	$LHMPC_{w\text{-}pHa}$	$MHMPC_{w\text{-}pHa}$	$LHMPC_p$	$MHMPC_p$
Protein (%, w/w)	1.1^{aA}	0.9^{bA}	1.1^{aA}	0.7^{bC}	1.1^{aA}	0.8^{bB}
Protein (% milk protein)	27.3^{aA}	21.6^{bA}	27.1^{aA}	18.6^{bC}	26.2^{aA}	19.9^{bB}
Casein (%, w/w)	0.4^{bA}	0.5^{aA}	0.4^{aA}	0.4^{aAB}	0.2^{aB}	0.3^{aB}
Csasein (% milk casein)	12.5^{aA}	15.0^{aA}	12.4^{aA}	12.7^{aAB}	6.7^{aB}	8.3^{aB}
Ca (mg/100 g)	16^{aB}	11^{bB}	-	-	39^{aA}	35^{aA}
Ca (% milk Ca)	13.1^{aB}	9.1^{bB}	-	-	26.2^{aA}	24.5^{aA}
P (mg/100 g)	11^{aB}	10^{aB}	-	-	38^{aA}	36^{aA}
P (% milk P)	16.5^{aB}	15.5^{aB}	-	-	36.7^{aA}	37.2^{aA}

[1]Sample codes: Low-heat and medium-heat milk protein concentrate powders (LHMPC, MHMPC), manufactured from low heat-treated (72°C for 15 s) or medium heat-treated (85°C for 30 s) skim milk, were dispersed in water ($LHMPC_w$, $MHMPC_w$) or milk permeate ($LHMPC_p$, $MHMPC_p$). Sub-samples of $LHMPC_w$ and $MHMPC_w$ were pH adjusted to pH 6.65 and denoted as $LHMPC_{w\text{-}pHa}$ and $MHMPC_{w\text{-}pHa}$, respectively.

[2]Serum phase of protein dispersions, obtained on ultracentrifugation at 100,000 × *g* at 25 °C.

[3] Values in a row relating to effect of milk heat treatment ($LHMPC_w$ and $MHMPC_w$, $LHMPC_{w\text{-}pHa}$ and $MHMPC_{w\text{-}pHa}$, and $LHMPC_p$ and $MHMPC_p$) and not sharing a common superscripted lower-case letter differ significantly ($P < 0.05$); values in a row relating to the effect of solvent ($LHMPC_w$, $LHMPC_{w\text{-}pHa}$ and $LHMPC_p$; and $MHMPC_w$, $MHMPC_{w\text{-}pHa}$ and $MHMPC_p$) and not sharing a common superscripted upper-case letter differ significantly ($P < 0.05$).

[4]WP, whey protein; NPN, non-protein nitrogen; TN, total nitrogen; Ca, calcium;P, phosphorus; -, denotes not determined.

7.3.3. Composition of protein dispersions

The composition of the MPC protein dispersions are shown in Table 7.2. Increasing the heat treatment of the skim milk prior to MPC manufacture did not significantly affect the gross composition or pH of the dispersions, as expected because of the similar compositions of the LHMPC and MHMPC powders. However, it led to a reduction in the concentrations of [Ca^{2+}] and serum Ca ($P < 0.05$) in the permeate-based dispersion ($MHMPC_p$) and of serum Ca in the water-based dispersion ($MHMPC_w$). The reduction in serum Ca suggests precipitation of serum Ca and P as colloidal calcium phosphate (CCP).

The use of permeate, rather than water, as solvent led to notable changes in gross composition, with the permeate-based dispersions ($LHMPC_p$ and $MHMPC_p$) having significantly higher concentrations of TS, lactose, total protein, NPN, Ca, P, [Ca^{2+}], serum Ca and serum P than the corresponding water-based dispersions ($LHMPC_w$ and $MHMPC_w$) (Table 7.2 and 7.3). The higher levels of the compounds in the MPC_p dispersions is consistent with their presence in the permeate (Table 7.1). In contrast, the pH of the $LHMPC_p$ and $MHMPC_p$ dispersions (~6.65) was ~0.35 units lower than that of the respective $LHMPC_w$ and $MHMPC_w$ dispersions, an effect most likely due to the lower pH of the permeate *per se*, and the presence of salts in the permeate (e.g., NaCl, KCl, sodium phosphate, sodium citrate) which promote dissociation of carboxyl groups on amino-acid residue side-chains of the caseins and the resulting release of protons.

Sub-samples of $LHMPC_w$ and $MHMPC_w$ were adjusted to a pH value (~ 6.65) similar to that of $LHMPC_p$ and $MHMPC_p$. The [Ca^{2+}] of the resulting pH-adjusted dispersions, $LHMPC_{w\text{-}pHa}$ and $MHMPC_{w\text{-}pHa}$, was significantly higher than that of $LHMPC_w$ and $MHMPC_w$, and that of $LHMPC_p$ and $MHMPC_p$. The decrease in pH

coincides with an increase in the solubilization of micellar calcium, as reflected by the increases in serum Ca and P.

7.3.4. Protein profile of the serum

Increasing the heat treatment of the milk from 72 to 85°C resulted in a lower concentration of protein in the serum phase of the $MHMPC_w$ and $MHMPC_p$ dispersions ($P < 0.05$), reflecting the heat-induced interaction of denatured whey proteins with the casein micelle (Singh & Creamer, 1991). Simultaneously, heat-induced dissociation of casein increased in the water-based dispersions, as evidenced by the higher concentration of non-sedimentable casein in the serum of $MHMPC_w$ compared to $LHMPC_w$. The increase in casein dissociation with intensity of heat treatment concurs with the findings of previous studies on (reconstituted) skim milk (Lin et al., 2017). Reducing the pH of the $MHMPC_w$ dispersion from 7.0 to 6.65 led to reductions in concentrations of protein in the serum, suggesting the re-association of non-sedimentable casein and/or κ-casein-whey protein aggregates with the casein micelle. Previous studies have shown that heat-denatured whey proteins complex with dissociated κ-casein to form serum-soluble particles/aggregates and this occurs to a greater extent as the pH during heating is increased over the range 6.5–7.5 (Ménard et al., 2005; Lin et al., 2017).

The substitution of water with permeate did not affect the total concentration of protein in the serum but resulted in a lower concentration of non-sedimentable casein and a higher concentration of NPN (Table 7.2 and 7.3). The lower concentration of non-sedimentable casein on using permeate is analogous to that found on addition of $CaCl_2$ to sodium caseinate dispersions (Pitkowski et al., 2009;

Sandra et al, 2013), whereby calcium addition contributed to association and assembly the caseins.

For all dispersions ($LHMPC_w$, $LHMPC_p$, $MHMPC_w$, $MHMPC_p$), the levels of individual caseins in the serum, as a proportion of the total corresponding casein in milk, was highest for κ-casein and lowest for α_{s1}-casein (Figure 7.1). Increasing milk heat treatment led to a greater increase in the degree of κ-casein dissociation in the water-based dispersions than in the permeate-based dispersions, as seen by comparing $LHMPC_w$ and $MHMPC_w$, and $LHMPC_p$ and $MHMPC_p$, respectively (Figure 7.1). The use of permeate instead of water significantly reduced the proportions of κ-, α_{s2}- and β-caseins in the LHMPC dispersions ($P < 0.05$), and of κ-, β- and α_{s1}-caseins in the MHMPC dispersions ($P < 0.05$; Figure 7.1). Reducing the pH of the $MHMPC_w$ dispersion from ~7.0 to 6.65 resulted in a higher [Ca^{2+}], lower zeta potential, a lower concentration of non-sedimentable casein, and lower proportions of non-sedimentable κ-, β- and α_{s1}-caseins in $MHMPC_{w\text{-}pHa}$ ($P < 0.05$). A similar trend was observed on reducing the pH of the $LHMPC_w$ dispersion, except that the change in concentration of non-sedimentable casein was not significant ($P > 0.05$, Tables 7.2 and 7.3).

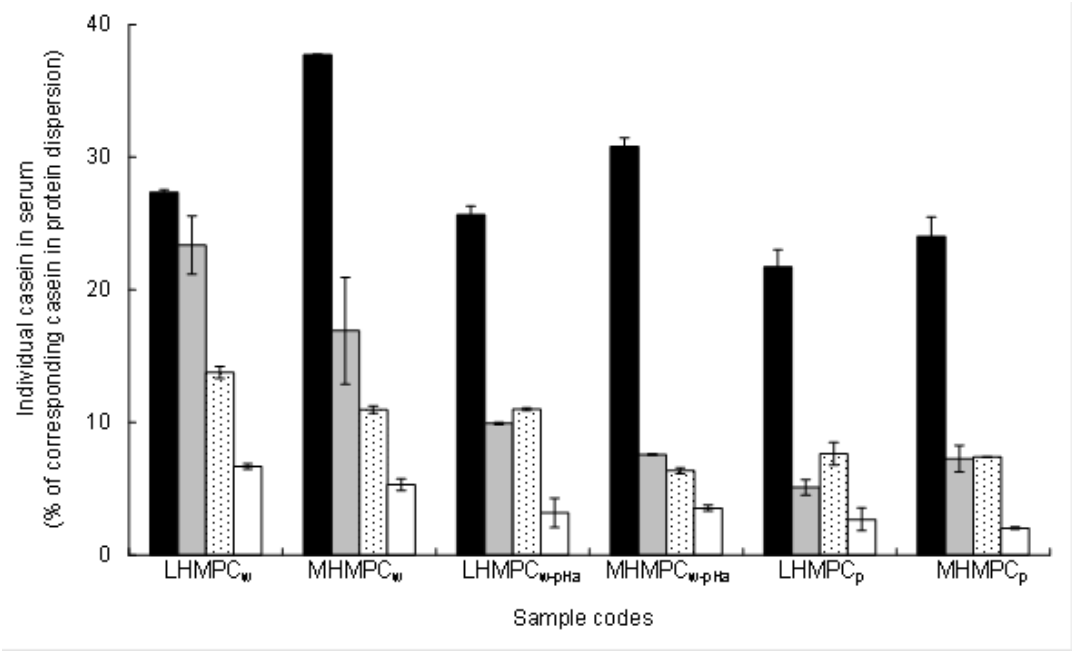

Figure 7.1. Proportions of individual caseins in the serum prepared by ultracentrifugation of the low-heat (LH) or medium-heat (MH) treated milk protein concentrate powders (MPC) reconstituted in water ($LHMPC_w$ and $MHMPC_w$), water followed by pH-adjustment to 6.65 ($LHMPC_{w\text{-}pHa}$ and $MHMPC_{w\text{-}pHa}$), or milk permeate ($LHMPC_p$ and $MHMPC_p$): κ- (■), α_{s2}- (▩), β- (▨) and α_{s1}- (□) caeins. Presented values are the mean values of duplicate trials; error bars represent standard deviations of the mean.

7.3.5. Physico-chemical properties

Increasing milk heat treatment coincided with an increase in particle size in both the MPC_w and MPC_p dispersions ($P < 0.05$), but did not affect casein hydration or zeta potential (Table 7.2). The increase in particle size is consistent with heat-induced denaturation of whey proteins and their interaction with κ-casein & thiol-disulphide interchange at the micelle surface (Singh et al., 1988; Corredig & Dalgleish, 1996; Anema et al., 2004).

The use of permeate in place of water significantly reduced particle size and zeta potential ($P < 0.05$), as seen by comparing $LHMPC_w$ and $LHMPC_p$, and $MHMPC_w$ and $MHMPC_p$, respectively (Table 7.2). The lower particle size and zeta

potential in the $LHMPC_p$ and $MHMPC_p$ dispersions is consistent with their lower pH, higher [Ca^{2+}], and the higher concentration of other ionic species such as K^+, Na^+, Mg^{2+} in the permeate (Table 7.1; Schmidt & Poll, 1986; Udabage et al., 2000; Philippe et al., 2003). Hence, when the pH of the $LHMPC_w$ and $MHMPC_w$ dispersions were reduced from ~7.0 to 6.65, the concentration of [Ca^{2+}] increased and both zeta potential and particle size decreased (Table 7.2). The reduction in particle size on reducing the pH of the water-based dispersions from 7.0 to 6.65 concurs with the findings of Sinaga et al. (2017) and reflects the decrease in zeta potential.

7.3.6. Rennet gelation of protein dispersions

Increasing the milk heat treatment during the manufacture of MPC led to a significant deterioration in rennet-coagulability, as evidenced by the inferior rennet coagulability (lower GFR_{max} and lower G'_{60}) of the $MHMPC_w$ and $MHMPC_p$ compared to $LHMPC_w$ and $LHMPC_p$, respectively (Figures 7.2a,b). The adverse effect of high heat treatment on rennet gelation has been widely reported for milk (Guinee et al., 1997; Schreiber, 2001). Contributing factors include the reduction in the [Ca^{2+}] (Singh et al., 1988; Schreiber, 2001), and the presence of the denatured whey protein-κ-casein aggregates at the micelle surface or in the serum which provide a steric hindrance to close approach and fusion of *para*-casein micelles (Ménard et al., 2005; Kethireddipalli et al., 2010; Lin et al., 2017).

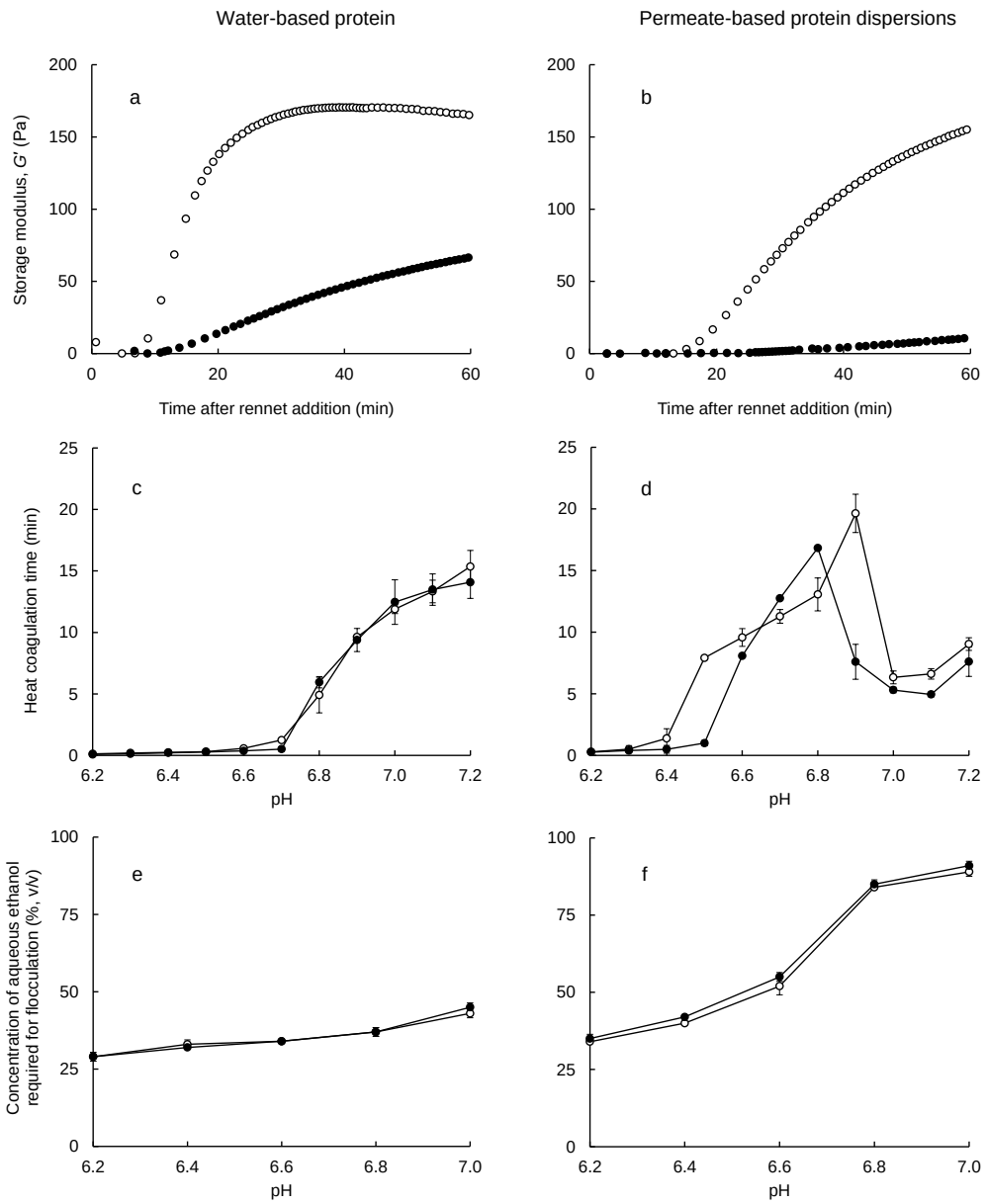

Figure 7.2. Effect of milk heat treatment (low-heat, open symbols; medium-heat, closed symbols) during the manufacture of milk protein concentrate (MPC) powder on rennet gelation characteristics (a, b), heat coagulation time (c, d) and ethanol stability (e, f) of milk protein dispersions prepared by reconstituting the MPC powder in water (a, c, e) or milk permeate (b, d, f).

The use of permeate instead of water led to a notable deterioration in rennet coagulability, as shown by the longer RGT and lower GFR_{max} of $LHMPC_p$ relative to $LHMPC_w$, and the lower GFR_{max} and G'_{60} of $MHMPC_p$ relative to $MHMPC_w$

(Figures 7.2a,b; Table 7.4). An opposite trend might be expected, considering the concomitant reductions in zeta-potential and particle size. The negative effect of substituting water with permeate on rennet gelation is most likely associated with the concomitant reduction in [Ca^{2+}], as seen by comparing $LHMPC_p$ with $LHMPC_{w\text{-}pHa}$, and $MHMPC_p$ with $MHMPC_{w\text{-}pHa}$ (Table 7.2), and the increase in concentration of soluble salts (e.g., NaCl, KCl, $MgCl_2$, NaCitrate; Table 7.1). The higher concentrations of soluble salts and ionic strength of the serum phase of the MPC_p dispersions is likely to promote a salting-in effect of the casein in the MPC_p dispersions, and thereby diminish casein-casein interaction by charge-screening (Damodaran, 1997) and reduce the rennet coagulability relative to the MPC_w dispersions (Abou El Nour, 1998).

The strong rennet gelation behavior of the $LHMPC_w$ and $MHMPC_w$ contrasts with the observations of Martin et al. (2010), who reported the failure of an aqueous dispersion of an experimentally-produced MPC in water (3.5% protein) to undergo rennet-induced gelation. The inter-study discrepancy may be related to differences in the concentrations of Ca, P and [Ca^{2+}] and pH at rennet gelation. The use of cold UF-DF (10°C) by Martin et al. (2010), compared to warm UF/DF at 50°C in the current study, is likely to have reduced the concentration of [Ca^{2+}] and CCP in the MPC (Brule & Fauquant, 1981; Law & Leaver, 1988; Eshpari et al., 2015). Hence, Martin et al. (2010) found that the addition of $CaCl_2$ at concentrations of 2-3 mM to the aqueous-based MPC restored rennet-induced gelation. It is also likely that the non-pH-adjustment of an aqueous-based MPC dispersion (e.g., to pH 6.55–6.60) prior to rennet addition would attenuate its rennet-induced coagulability (Nájera et al., 2003).

Table 7.4. Rennet-induced gelation and model stirred skimmed yoghurt-making characteristics of milk protein dispersions.[1,2,3,4]

	Water-based protein dispersion		Permeate-based protein dispersion	
	$LHMPC_w$	$MHMPC_w$	$LHMPC_p$	$MHMPC_p$
Rennet-induced gelation				
RGT (min)	6.9^{bB}	9.9^{aA}	13.9^{bA}	18.9^{aA}
GFR_{max} (Pa/min)	233^{aA}	46^{bA}	93^{aB}	14^{bB}
G'_{60} (Pa)	156^{aA}	74^{bA}	156^{aA}	14^{bB}
Gelation during yoghurt manufacture				
Denatured WP (%total WP)	79.7^{aA}	82.3^{aA}	76.3^{aA}	81.7^{aA}
GOT_{pH}	5.62^{aA}	5.56^{aA}	5.20^{aB}	5.19^{aB}
GOT (min)	182^{aA}	187^{aA}	149^{aB}	150^{aB}
$G'_{pH4.6}$ (Pa)	470^{aA}	316^{bA}	171^{aB}	108^{aB}
Time to reach pH 4.6 (min)	450^{bA}	470^{aA}	205^{aB}	210^{aB}
Yoghurt properties				
σ_o (Pa)	3.4×10^{-8aA}	1.7×10^{-7aA}	4.9×10^{-8aA}	0.3^{aA}
K (Pa s^n)	8.8^{aA}	6.5^{aA}	8.2^{aA}	1.2^{bB}
n	0.26^{aA}	0.25^{aA}	0.20^{aA}	0.43^{aA}
η_{10} (mPa.s)	1447^{aA}	1082^{bA}	1263^{aB}	642^{bB}
η_{120} (mPa.s)	241^{aA}	175^{bA}	170^{aB}	100^{bB}
WHC (g serum retained/100 g)				
300 × *g*	66.8^{aA}	66.7^{aA}	66.7^{aA}	65.2^{aA}
2500 × *g*	38^{aA}	35^{aA}	34^{aA}	35^{aA}

[1]Sample codes: Low-heat and medium-heat milk protein concentrate powders (LHMPC, MHMPC), manufactured from low heat-treated (72°C for 15 s) or medium heat-treated (85°C for 30 s) skim milk, were dispersed in water ($LHMPC_w$, $MHMPC_w$) or milk permeate ($LHMPC_p$, $MHMPC_p$).

[2]Values in a row relating to effect of milk heat treatment during manufacture of milk protein concentrate ($LHMPC_w$ and $MHMPC_w$, and $LHMPC_p$ and $MHMPC_p$) and not sharing a common superscripted lower-case letter differ significantly ($P < 0.05$); values in a row relating to the effect of the solvent used for dispersion of MPC ($LHMPC_w$ and $LHMPC_p$; and $MHMPC_w$ and $MHMPC_p$) and not sharing a common superscripted upper-case letter differ significantly ($P < 0.05$).

[3]RGT, rennet gelation time; GFR_{max}, maximum gel firming rate; G'_{60}, storage modulus at 60 min; WP, whey protein; GOT_{pH}, gelation onset pH; GOT, gelation onset time; $G'_{pH4.6}$, storage modulus at pH 4.6; σ_o, yield stress; K, consistency coefficient; n, flow behaviour index; η_{10}, viscosity of sample at shear rate of 10 (1/s); η_{120}, viscosity of sample at shear rate of 120 (1/s); WHC, water holding capacity.

[4]WHC, water holding capacity, was the quantity of serum retained by the yoghurt followingcentrifugation at 300 or 2500 × *g*.

7.3.7. Heat coagulation time of protein dispersions

Increasing the severity of milk heat treatment from 72°C for 15 s to 85°C for 30 s during MPC manufacture had no effect on the HCT of the water-based MPC dispersion as a function of pH in the range 6.2–7.2 (Figure 7.2c). However, in the case of the permeate-based MPC dispersion (Figure 7.2d), it reduced the pH of HCT_{max} from ~6.9 to 6.8 and the HCT at pH values 6.4, 6.5 and 6.9 ($P < 0.05$). The latter effect is typical of the trend reported for the effect of increasing heat treatment on the HCT *vs.* pH profile of reconstituted milk powder (Carr, 1999; Lin et al., 2017).

The solvent system had a marked effect on the shape of HCT *vs*. pH curves as seen by comparing the profiles of $LHMPC_w$ and $MHMPC_w$ with $LHMPC_p$ and $MHMPC_p$, respectively. Most notably, the replacement of water with permeate changed the HCT *vs.* pH profile from a sigmoidal shape, where HCT remained essentially constant at pH values 6.2–6.7, and increased curvilinearly at a diminishing rate in the pH region 6.8–7.2 (Figure 7.2c), to the more typical type A curve exhibiting a HCT_{max} and HCT_{min} (Figure 7.2d). The HCT *vs*. pH profile of the water-based MPC dispersion (Figure 7.2c) is similar to that reported by Crowley et al. (2014). Le Ray et al. (1998) reported the heat stability of an aqueous dispersion of phosphocasein (pH 6.67) at 95°C was significantly improved on substitution of water with milk permeate. Based on the analysis of the protein dispersions and their sera (Tables 7.2 and 7.3), the relatively low HCT of the water-based dispersions ($LHMPC_{w\text{-}pHa}$ and $MHMPC_{w\text{-}pHa}$) at pH 6.2–6.7 may be due to their relatively high [Ca^{2+}] and degree of κ-casein dissociation (Figure 7.1), while the relatively high HCT at pH 6.8–7.2 may be associated with their higher zeta potential and lower [Ca^{2+}] (Table 7.2). However, the presence of a greater range of salts (e.g., citrate, KCl) and higher concentrations of soluble salts (e.g., phosphate) in the permeate-

based dispersions is also likely to alter the HCT *vs.* pH profile from that in the water-based dispersions (Augustin & Clarke, 1990). It is noteworthy that Fox and Hearn (1978) found type A milk was converted to type B milk on partial demineralization by dialysis against water.

7.3.8. Ethanol stability of protein dispersions

The ES of MPC dispersions are shown in Figures 7.2e,f. Altering the heat treatment of the skim milk during MPC manufacture had little, or no, effect on the ES of the MPC_w or MPC_p dispersions over the pH range 6.2–7.0. This trend contrasts with previous studies which reported a significant increase in ES of skim milk over the same pH range on high heat treatment of the milk, e.g., at 90°C for 30 min or 120°C for 2–30 min (Horne & Parker, 1981a; Lin et al., 2017), and attributed the increase to the heat-induced reduction in $[Ca^{2+}]$. The inter-study discrepancy may be related to the intensity of heat treatment and its impact on the change in $[Ca^{2+}]$ of the reconstituted MPC powder with pH.

Solvent composition had a major influence on ES, as shown by the markedly lower ES of the water-based dispersions, $LHMPC_w$ and $MHMPC_w$, compared to the permeate-based dispersions, $LHMPC_p$ and $MHMPC_p$, especially at pH 6.6–7.0. The detrimental effect of substituting permeate with water on the ES of MPC dispersions is indicative of a destabilization and aggregation of casein micelles (Horne, 2016), which is consistent with the associated increases in $[Ca^{2+}]$ and κ-casein dissociation (Table 7.2, Figure 7.1). It is noteworthy that the addition of NaCl to milk has been found to promote κ-casein dissociation (Tessier & Rose, 1964) and reduce ES in the pH region 6.6–7.0 (Horne & Parker, 1981b), which coincides with the region of the

largest difference between the ES of the MPC_w and MPC_p dispersions (Figures 7.2e,f).

7.3.9. Model stirred skimmed yoghurt

Acidification and gel-formation. The changes in pH and G′ during the acidification and gelation of MPC dispersions from Trial 1 are shown in Figure 7.3; similar changes were observed in Trail 2 (data not shown).

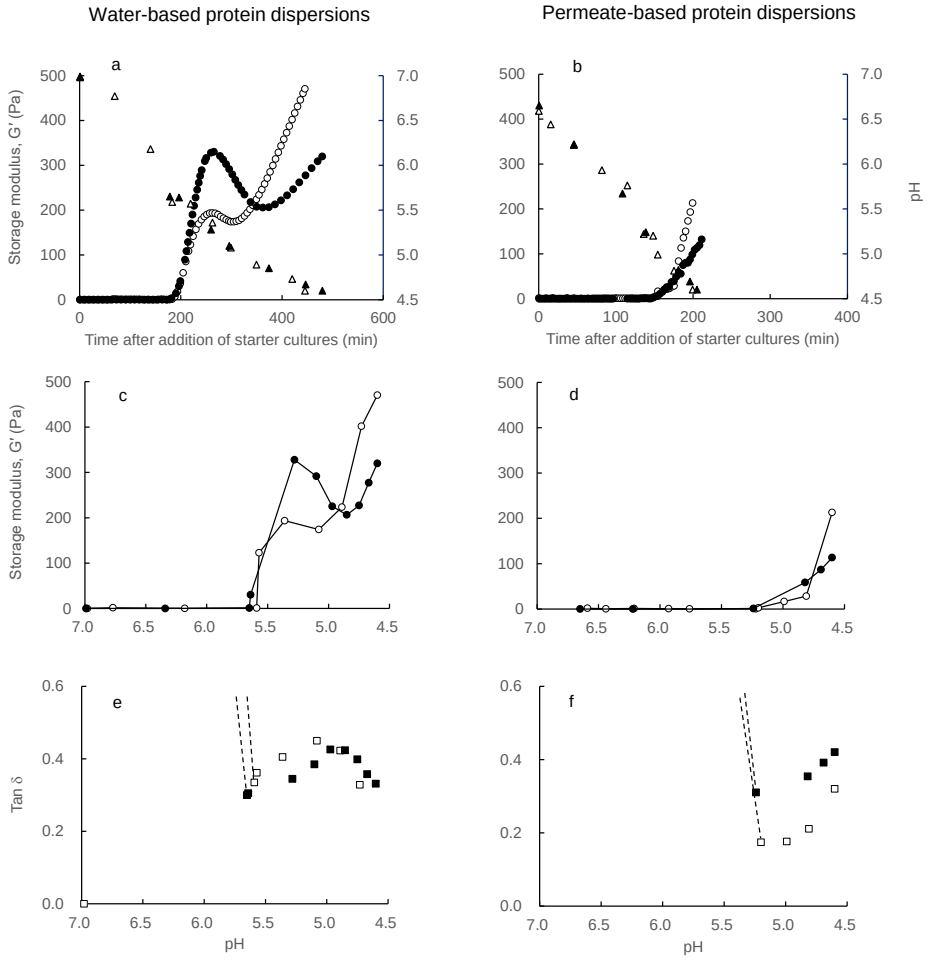

Figure 7.3. Effect of milk heat treatment (low-heat, open symbols; medium-heat, closed symbols) during the manufacture of milk protein concentrate (MPC) powder on the gelation characteristics of model stirred skimmed yoghurt from protein dispersions (5% protein) prepared by reconstituting the MPC powder in water (a, c, e) or milk permeate (b, d, f): changes in pH (△,▲) and storage modulus, G′ (○,●) as functions of time (a, b), and storage modulus, G′ (c, d) and tan δ (e, f) as functions of pH. Broken lines indicate that tan δ increased to values >> 1.0.

Solvent composition had a major influence on the time to reach pH 4.6 (Table 7.4), the profile of the G' *vs.* time curve, gelation-onset time (GOT) and G' at pH 4.6 ($G'_{pH4.6}$) for both the LHMPC and MHMPC dispersions (Figure 7.3). The water-based dispersions ($LHMPC_w$, $MHMPC_w$) required a significantly longer time (~200 min) to reach the target pH (pH 4.6), which is consistent with their higher initial pH (i.e., ~7.0 versus 6.65 for the permeate-based dispersions). Other factors contributing to the longer gelation time in the water-based dispersions may include the absence of minerals and vitamins that are normally present in milk serum and which are required for, or stimulate, the growth of starter bacteria (Hayek & Ibrahim, 2013).

Compared to the permeate-based dispersions ($LHMPC_p$, $MHMPC_p$), the water-based dispersions had a longer GOT, a higher pH at gelation onset (GOT_{pH}), a higher $G'_{pH4.6}$ (Table 7.4, Figure 7.3), and were characterized by an inflection point (peak), which occurred at an advanced stage of gelation, i.e., ~70–100 min after the GOT (Figures 7.3a-b). This was also observed from plots of tanδ and G′ as functions of pH (Figures 7.3c–f). Hence, G' increased following gelation onset at pH ~5.6 in the water-based dispersion, then decreased abruptly to an extent dependent on the type of MPC ($LHMPC_w$ or $MHMPC_w$) and thereafter increased again as the pH decreased to 4.6. A similar, though less pronounced peak was also observed by Meletharayil et al. (2015) during the GDL-induced gelation of an aqueous dispersion of MPC80, but not in dispersions from MPC powders with 50–70% or 85–90% protein. No such inflection point was observed for G' *vs.* time, G' *vs.* pH, or tanδ *vs.* pH in the permeate-based dispersions, for which G' increased and tanδ progressively from GOT at pH ~ 5.2.

The presence of an inflection point in the G' *vs.* time, G' *vs.* pH, or tanδ *vs.* pH curves of the water-based MPC dispersions would appear to be a more extreme form of the 'shoulder' feature observed during acid-induced gelation of high-heat treated milk (Lucey, 2016). The shoulder has been ascribed to the competitive effects of two physico-chemical changes with opposite effects of G', namely a pH-induced aggregation of denatured β-Lg (complexed with the κ-casein at the micelle surface) which enhances micelle aggregation, and solubilisation of CCP which promotes hydration and disaggregation of casein (Meletharayil et al., 2015; Lucey, 2016). The occurrence of the inflection point in the G' *vs.* time or pH profiles of the water-based dispersions and its absence in the corresponding permeate-based dispersions cannot be attributed to differences in whey protein denaturation, which was similar (~76–82% of total) for both (Table 7.4). Instead, it may reflect differences between the dispersions with respect to parameters that influence the degree and type of structural-rearrangements within the gel, e.g., proportions of serum Ca, P (Table 7.3), CCP (Anema, 2009) and rate of pH reduction. Differences between the water- and permeate-based dispersions with respect to the course of tanδ with pH from GOT supports a greater potential for bonds and strands within the gel from the water-based dispersions to relax and, thereby facilitate more rearrangement of the gel and higher ultimate gel strength, i.e., G' (Lucey, 2016).

The relatively high GOT_{pH} for the water-based dispersions (~ 5.6 *vs.* 5.2 for the permeate-based dispersions) may reflect their higher pH (i.e., ~7.0 compared to ~6.65 for the permeate-based dispersion) of the milk at the heat treatment applied during yoghurt manufacture (Table 7.2). The higher pH of the water-based dispersion during heat treatment is conducive to greater dissociation of κ-casein and the formation of serum-soluble complexes of κ-casein and denatured whey proteins

(Vasbinder & de Kruif, 2003; Ménard et al., 2005; Lin et al., 2017). Similarly, previous studies have reported a marked increase in the GOT_{pH} of GDL-induced gels prepared from reconstituted skim milk powder when the pH of the skim milk at heat treatment was increased from 6.2–6.5 to 6.9–7.1, prior to cooling, fermentation and gelation (Vasbinder & de Kruif, 2003; Anema et al., 2004; Lakemond & van Vliet, 2008). The higher GOT_{pH} of the water-based dispersions probably reflects their higher pH (i.e., ~7.0 compared to ~6.65 for the permeate-based dispersions, Table 7.2) and its influence on the partitioning of κ-casein (Figure 7.1) and denatured whey protein between the serum and the micelle. The proportion of denatured whey protein associated with the casein micelle decreases as the pH of milk at heating is increased from 6.6 to 6.9–7.5 (Vasbinder & de Kruif, 2003; Ménard et al., 2005; Lin et al., 2017). Anema et al. (2004) hypothesised that the serum-soluble denatured whey protein-κ-casein aggregates in milk heated at high pH may gel separately from the casein micelles. As the isoelectric pH of the serum-soluble aggregates (pH ~ 5.3) is higher than that of the casein micelles (pH ~ 4.6), the pH at which gelation occurs shifts to higher pH as the pH of the milk at heating increases. Alternatively, Lakemond and van Vliet (2008) suggested that the higher GOT_{pH} of acid gels from milk heat treated at higher pH values in the range 6.2 to 6.9, was associated with a number of changes, that affect particle aggregation and rearrangements processes before and just after gelation, including the structure of the casein micelle surface, extent thiol-interactions, and the size of heat-induced complexes. κ-Casein dissociation led to a smoother micelle surface (i.e., more devoid of protruding κ-casein) and less steric hindrance to the close approach of, and earlier bonding between, the casein micelles during acidification.

Rheological properties. The shear rate *vs*. shear stress data for all yoghurts fitted to the Herschel-Bulkley model ($R > 0.99$). All yoghurts exhibited a yield stress and shear thinning behaviour (Figures 7.3c,d), reflecting the presence of internal casein-whey protein network which was disrupted during shearing. However, the values of σ_o were low ($< 1.7 \times 10^{-7}$ to 0.3 Pa) and did not differ between yoghurts ($P > 0.05$). Overall, the $LHMPC_w$ dispersion had the highest viscosity during shearing and the $MHMPC_p$ the lowest.

Increasing the severity of milk heat treatment prior to MPC manufacture had no effect on the value of consistency coefficient, K, but led to a significant reduction in viscosity over the entire shear rate range for both the water- and permeate-based dispersions (Table 7.4). This trend and the similar levels of denatured whey protein in all yoghurt milks (Table 7.4), suggests that partial pre-denaturation of whey protein during MPC manufacture (e.g., ~ 48 % in the MHMPC powder) reduce the viscosity of model stirred skimmed yoghurt.

The use of permeate instead of water had effects similar to those obtained on increasing the severity of milk heat treatment during MPC manufacture, except that it led to a reduction in the K value for the $MHMPC_p$ yoghurt (Table 7.4). The effect of solvent may relate to differences in the rate of different physico-chemical changes occurring during gel formation, namely solubilisation of CCP and reduction in the charge of proteins, and their impact on network formation and rearrangement prior to end of yoghurt manufacture (Lucey, 2016).

Water holding capacity. The WHC of the yoghurt decreased as centrifugation force was increased from 300 to 2500 × *g* (Table 7.4). It was unaffected by heat treatment of the skim milk during MPC manufacture or by using milk permeate

instead of water as solvent ($P > 0.05$; Table 7.4). The results suggest that the impact of any differences in yoghurt viscosity and microstructure due to skim milk heat treatment during MPC manufacture or solvent composition on WHC may have been overcome owing to structural collapse at the centrifugation forces applied (Harwalkar & Kaláb, 1986).

7.4. Conclusion

This study investigated the effects of milk heat treatment during the manufacture of MPC powder (~81% protein) and the solvent used for reconstitution on the composition, physico-chemical properties and functionality of the resultant MPC dispersions. The milk heat treatment during MPC manufacture affected rennet gelation, heat coagulation time as a function of pH at 6.2–7.2 and gel formation properties and consistency of model stirred skimmed yoghurt to an extent dependent on the solvent composition. Ethanol stability was affected by solvent composition, but not by heat treatment during MPC manufacture. These effects were associated with differences in whey protein denaturation between the MPC powders, and in the composition, degree of κ-casein dissociation, particle size and charge, and ionic calcium content of the resultant MPC dispersions. The results highlight the importance of the severity of milk heat treatment during the manufacture of MPC and the composition of the solvent used for reconstitution of the MPC powder when formulating beverages or semi-solid food products. This study also highlights the need for model studies on the systematic effect of increasing the type, level and combination of different components (such as salts, sugars) on the properties of aqueous-based MPC dispersions. The information gleaned should provide a more

systematic insight into food formulation and the factors affecting protein aggregation in aqueous-based MPC dispersions.

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Chapter 8

General discussion

Protein, the commercially most important component in milk, is widely used in food formulations and dairy-based products to harness its nutritional and functional properties. Critical protein aggregation characteristics include the stability of protein to rennet, heat, ethanol and acid, which are closely associated with the functional properties of milk and dairy ingredients. These functional properties are required in food applications such as rennet-curd cheese, high-protein beverages, recombined milk, alcohol-based beverages and yogurt (Agarwal et al., 2015; Sharma et al., 2012). The current study investigated the changes in composition, physico-chemical and functional properties of skim milk due to seasonality, manufacturing conditions including heat treatment, evaporation and drying during skim milk powder manufacture, concentration of skim milk, addition of milk protein ingredients and the level of ingredients added to skim milk, heat treatment of skim milk during the manufacture of milk protein concentrate (MPC), and composition of solvent used for reconstitution of the MPC powder.

8.1 Fortification of milk protein

The results from the study on seasonal variation of skim milk showed the changes in volume proportion of spring-calving milk (% of total milk) with season, being highest in Summer and lowest in Winter (Chapter 2). The results of compositional and physico-chemical analysis characterized the seasonal variation in milk composition, such as levels of protein, calcium (Ca) and phosphorus (P), which has a marked influence on functional properties of skim milk. For example, to reduce or eliminate the impacts of seasonal variations in protein functionalities, standardization of milk protein in cheese milk using skim milk powder (SMP) is essential and commonly used in cheese manufacture such as Cheddar and

Mozzarella. A possible approach to standardize protein content of milk in cheese manufacture or that of high-protein beverages is to add protein ingredients, such as low-heat SMP, sodium caseinate (NaCas), calcium caseinate (CaCas), native phosphocasein and calcium-reduced phosphocasein. The effect of type and level of milk protein ingredients used in fortification of milk protein content were examined (Chapter 3 and 4).

The type of dairy protein powder used to fortify the protein content of skim milk significantly affected rennet gelation, heat stability, and ethanol stability. The skim milk or skim milk fortified with micellar caseins (e.g., phosphocasein) contains micellar (native) casein micelles while some of the fortified milk samples (e.g., NaCas, CaCas, Ca-reduced phosphocasein) contain a mixture of micellar casein micelles and non-micellar casein. The influence of ingredient type was mainly related to its degree of mineralization (e.g., calcium and phosphorus), the state of added protein (micellar or non-micellar), and its effects on the ionic calcium ($[Ca^{2+}]$) content and proportions of non-sedimentable casein and non-sedimentable calcium (Ca) in the fortified milk. The non-sedimentable casein in some fortified milk such as NaCas fortified milk may consist of native non-sedimentable casein and non-sedimentable casein from NaCas. The use of NaCas, CaRPC and CaCas for protein fortification of skim milk from 3.3 to 4.1% protein resulted in inhibited rennet gelation, higher heat coagulation time (HCT) in the pH range 6.7-7.2, and enhanced ethanol stability. Further investigation ot the level of NaCas used for fortification of milk protein indicated that the addition of NaCas, even at low levels (0.2-0.4%), significantly impaired the rennet gelation of skim milk, while simultaneously enhancing heat stability in the pH range 6.7-7.2 and ethanol stability at pH 6.4. These effects were primarily associated with an increase in the proportions of non-

sedimentable casein and Ca, and a reduction in the concentration of [Ca^{2+}]. It was hypothesised that the negative effect of NaCas on rennet gelation was due to this increase in non-sedimentable casein, which upon hydrolysis by chymosin forms small non-sedimentable particles that physically come between, and impede the aggregation of, rennet-altered *para*-casein micelles, and thereby inhibit the development of a gel network.

8.2 Effect of processing operations on milk protein dispersions

In Chapters 5-7, the effects of processing operations and conditions of reconstitution of milk powders were investigated; the skim milk powder was reconstituted to the same level of protein as in raw skim milk used in each study to eliminate the changes induced by protein content on functionalities of milk.

Evaluation of the different steps and their combined effect (milk heat treatment, evaporation, spray-drying) during the manufacture of SMP showed that the intensity of milk heat treatment was the dominant factor controlling the physico-chemical properties and functionality of the reconstituted SMP (Chapter 5). More severe heat treatment coincided with impaired rennet gelation, reduced the pH of maximum heat stability, increased the heat stability of skim milk concentrates at pH 6.3-6.5, and improved ethanol stability at pH in the range 6.2-6.6. The effects were associated with increases in whey protein denaturation, binding of denatured whey protein to the casein micelles, κ-casein dissociation, calcium phosphate precipitation and increased casein micelle size, and a reduction in concentration of [Ca^{2+}]. Another important factor that determined the effect of milk heat treatment on protein functional properties was the pH of milk at heating (Chapter 6); increasing the pH of

milk from ~6.6 to 7.2 or 7.5 prior to heat treatment, followed by restoration after heating, resulted in increases in casein dissociation and in the concentration of serum-soluble κ-casein-denatured whey protein aggregates, and reductions in casein micelle size and serum Ca. Simultaneously, the heat stability at pH intervals of 6.6–6.7 and 7.2 increased. Otherwise, altering pH before heating did not influence rennet gelation or ethanol stability of high-heated treated milk. The adverse effect on rennet gelation was attributed to the reduction in the concentration of serum Ca and the formation of κ-casein-denatured whey protein serum-soluble aggregates that hinder the fusion of the *para*-casein micelles. Increasing severity of milk heat treatment during the manufacture of MPC from 72°C for 15 s to 85°C for 30 s significantly impaired the rennet gelation properties of MPC dispersions (4.0% protein), but had little or no effect on heat stability, ethanol stability or acid-induced gelation (Chapter 7). The [Ca^{2+}] content is also affected by the temperature at ultrafiltration (Law & Leaver, 1998), which may cause the impaired rennet gelation in other studies (Martin et al., 2010).

Skim milk concentrates obtained on increasing the total solids during reconstitution of skim milk powder showed significantly increased and decreased rennetability and heat stability, respectively. However, high-heat treatment of milk prior to concentrating diminished the negative effect of higher total solids on heat stability due to denaturation of whey protein and precipitation of calcium phosphate (Chapter 5).

8.3 Effect of reconstitution conditions on milk protein dispersions

The environment that milk protein ingredients are exposed to varies during food manufacture and significantly affects the functional expression of milk protein powders (Crowley et al., 2014; Eshpari et al., 2015). Chapter 7 also examined the use of milk permeate instead of water as solvent for the preparation of MPC dispersions, which significantly affected their functionalities, as shown through increases in rennet gelation time and heat stability in the pH region of 6.3-.6.5, and decreased in the pH onset of acid gel. These effects were associated with differences in ionic strength (e.g., concentrations of Ca, P, magnesium, sodium, and potassium) of the solvent, which affected the degree of κ-casein dissociation, micelle size and charge, and concentration of [Ca^{2+}].

8.4 Overall conclusions

Overall, the research presented in this thesis highlights the fact that fortification of skim milk as a base using different protein ingredients, varying processing operations during the manufacture of milk powders, and the conditions of reconstitution of protein ingredients resulted in changes in compositional, physico-chemical and functional properties in the resultant material, many of which are interrelated.

Ca plays a fundamental role in the aggregation behaviour of milk proteins. Changes in calcium content and the partitioning of Ca between micellar and serum phases may be due to the seasonality, addition of protein ingredients, various Ca content in the protein ingredients and the form of casein micelle (e.g., micellar casein or non-micellar casein), total solids content of milk, heat treatment of milk, and the

type of solvent used for reconstitution of milk protein. Reductions in levels of serum Ca and [Ca^{2+}] resulted in reduction in the rennetability, increases in heat stability and ethanol stability. Apart from the serum Ca, the levels of Ca and P and the ratios of Ca:P are the fundamental factors for the mineralization in protein ingredients. Relatively low levels of Ca and P in protein ingredients (e.g., NaCas) yield lower levels of mineralization in the ingredient, giving lower levels of casein crosslinking density, hence, lower degree of micellization, forming a non-micellar casein ingredient. This is concomitant with increase in proportion of serum casein which may affect the aggregation of casein micelles by creating a physical barrier that impedes the knitting or collisions of casein micelles, thereby impairing the rennet gelation and enhancing the heat stability and ethanol stability of the NaCas-fortified skim milk.

Heat treatment regulated protein aggregation by inducing marked changes in the state of the proteins, milk serum composition and physico-chemical properties, evidenced by the whey protein denaturation, dissociation of κ-casein, reductions in serum Ca and [Ca^{2+}] and increases in casein micelle size. These changes are linked to the deterioration in rennet gelation, shift of pH at HCT_{max} to lower pH and the broadened pH range of HCT_{min}, and enhanced ethanol stability. The increase in level of denatured whey protein and reduction in [Ca^{2+}] are the main reasons for the higher stability of casein micelles to heat in concentrates prepared from high-heat-treated skim milk than that from low-heat treated skim milk.

Protein aggregation was also affected by components of the solvent in which the protein ingredients were rehydrated. The use of permeate instead of water for reconstitution of MPC dispersion significantly decreased the rennet-induced gel

forming rate, increased the ethanol stability and pH onset of acid gel, and modified the HCT profile from type B to A. The changes were attributed to the salt-in effect on protein in increase in ionic strength, in conjunction with the changes in [Ca^{2+}] and κ-casein dissociation.

In conclusion, the current study has provided insights in the factors and possible mechanisms influencing the rennet gelation, heat stability, ethanol stability and acid gelation, which is particularly relevant to the dairy industry where protein ingredients are required for their functional properties in food manufacture or used in food formulation.

8.5 Applications and practical relevance

The current results add knowledge in the use of appropriate approaches, including adjustment of pH prior to heat treatment, addition of protein ingredients at an appropriate level, and modification of solvent composition, for design and optimization of milk and dairy protein ingredients. This would be beneficial in applications of protein ingredients in dairy products and food formulations including cheese, recombined milk, protein beverages, cream liqueurs and yogurt.

The present study provides opportunities to improve the rennetability of milk with addition of native phosphocasein, which offers more options than ultrafiltration or addition of SMP for milk standardization in cheese manufacture. Replacement of SMP with native phosphocasein decreased the rennet gelation time and increases the rennet-gel firmness. These defects may be induced by excessive lactose from added SMP which can be used for protein standardization in cheese manufacture (Soodam & Guinee, 2018). Moreover, increasing the protein content of cheese milk using

micellar casein provides advantages in increased levels of fat recovery and normalised cheese yield (Guinee et al., 2006)

Furthermore, the current work opens the possibility of improving the heat stability of milk by addition of NaCas to skim milk or partial replacement of skim milk powder with NaCas in the formulation. The fortified milk has higher heat stability in the pH range from 6.7-7.2, which is favoured for high-protein beverages and recombined milk. A more heat-stable high-heat SMP may be produced by increasing the pH prior to heat treatment of milk, followed by restoration of pH, evaporation and spraying-drying. Moreover, adjustment of pH before heating could also be used in conjunction with novel separation techniques, and may provide an approach for development of new ingredients (e.g., κ-casein, β-lactoglobulin-enriched powders; κ-casein-depleted micelles). The improvement in ethanol stability in milk fortified with protein ingredient or heat-treated milk might enable the development of alcoholic-milk based beverages (e.g., cream liqueurs), with alcohol content ≥ 60%, manufactured at milk pH 6.6.

The current results also demonstrated the importance of controlled manufacture conditions of MPC. To retain the rennetability, low-heat treatment of milk should be preferred followed by warm ultrafiltration (~ 50°C). Attention should be drawn to the solvent selected for reconstitution of MPC matching the pH at heat treatment to avoid the heat coagulation, which is critical in formulation of high-protein beverages and ice cream. For example, MPC should be reconstituted in milk permeate, which gives high heat stability at native pH of milk during processing (pH 6.6-6.7).

8.6 Recommendations for future research

Based on the results that the hydrolysis of milk during renneting was not affected by addition of NaCas, it is hypothesized that the non-sedimentable casein may 'float' around the *para*-casein micelles and physically impede the aggregation process (Lin et al., 2016, 2017) or that absorption of NaCas may increase the steric repulsion between rennet-altered particles (Gaygadzhiev et al., 2012). It would be interesting to use transmission electron microscopy to track the NaCas in the rennet-treated milk, which may give more definite answers to as the role of NaCas during rennet gelation.

Addition of CaCas or Ca-reduced phosphocasein, which increased protein content of skim milk by 0.8%, resulted in inhibited rennet gelation and increased heat stability and ethanol stability (Lin et al., 2106). Further investigations on how different levels of CaCas and Ca-reduced phosphocasein affect the functional properties of milk would be beneficial in providing more choice of protein ingredients for fortification of milk protein.

The current study also emphasized the importance of levels of whey protein denaturation, and distributions of casein, Ca and P in functionalities of milk. It is essential to attempt to separate the multiple factors influencing the rennet gelation, heat stability, ethanol stability and acid gelation of milk in order to determine precisely which factor, or what combination of factors, are responsible for the changing functional properties in the resultant milk. In skim milk with different components naturally present, it is difficult to quantify the effect of any one particular component on functional properties of casein. The factors affecting the functional properties of milk protein include the type and concentrations of whey proteins (e.g., α-lactalbumin, β-lactoglobulin, and bovine serum albumin) and

minerals (e.g., Ca, P, sodium, potassium and magnesium), and distribution of whey proteins, Ca and P between micellar and serum phases. The effect of a single factor can be determined using a model system prepared from micellar casein (~2.8% casein), in which the concentrations and types of whey proteins and minerals can be controlled by addition of these components to micellar casein dispersion. The use of model systems subjected to different manufacturing conditions (e.g., heat treatment, pH adjustment, evaporation, dehydration), followed by the compositional analysis on (e.g., casein, individual whey proteins, Ca and P) in the micellar and serum phases of protein dispersions, may provide better understanding in the role of individual factors in the functional properties of milk.

Additionally, after understanding the causes of changes in functionality in model system containing ~2.8% casein content (similar level as in milk), the casein content then could be further increased to 7.4%, which stimulates the casein content in milk concentrate (~25% total solids). The same approach, such as addition of each constituent (whey proteins and minerals) at different levels individually or in combination, can be employed to study the effects of single factors or combination of factors on the functionality of concentrates. This may lead to a more controlled milk formulation which can be used specifically for recombined milk and high-protein beverages.

Last but not least, little is known about the direct compositional changes in milk when heat-induced coagulation or ethanol-induced flocculation occurs. To expand the knowledge on the mechanism of heat coagulation of milk, it is necessary to analyse the composition of the supernatants of the above model system which is heat treated at 140°C until the milk aggregates, in the pH range from 6.2 to 7.2. For ethanol stability, centrifugation of the model systems following precipitation induced

by addition of appropriate concentration of ethanol to the model system in the pH range from 6.2 to 7.0. The evaluation of the partition of protein and minerals (in particular Ca and P) between the sedimentable and non-sedimentable phases at different pH value may provide more information on process of coagulation induced by heat treatment or ethanol.

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Appendix

Publications in international peer-reviewed journals from this PhD project**.**