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Improving ultra high temperature processability of high protein beverages

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Improving Ultra High Temperature Processability of High Protein Beverages

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Abstract

Ultra-high temperature (UHT) stability of ready to drink (RTD) high milk protein beverages is mainly governed by their protein concentration, the relative abundance of the type of milk proteins present in their formulation and heat induced interaction of milk proteins among themselves and other beverage ingredients. The present PhD project was focused on addressing these issues in order to improve the UHT stability of high protein beverages.

The UHT (145 °C for 5 s) stability and fouling behaviour of reconstituted low heat skimmed milk powder (RSMP) and milk protein concentrate powder (RMPC) was compared to study the effect of type of milk protein ingredient and protein concentration on UHT stability of high protein beverages. RMPC at 10 and 14% protein content was more UHT stable as compared to lower protein content RSMP (3.25 to 8%). Matching the total solids and mineral composition of 7.5% protein RMPC with 7.5% protein RSMP by addition of minerals and lactose markedly reduced its UHT stability (UHT run-time reduced to 66 min from >120 min). The RP-HPLC analysis showed increased casein dissociation but similar whey protein aggregation in 7.5-RSMP as compared to 14-RMPC. UHT processing led to formation of larger particles in case of 7.5-RSMP (1.84 μm D(0.9)) as compared to 14-RMPC (0.23 μm D(0.9)). It was observed that mineral environment affected protein interactions leading to the differences in UHT behaviour of RSMP and RMPC.

The role of major minerals content in high heat stability of RMPC as compared to RSMP was further studied by matching Ca, Mg, Na and K contents of 7.5-RMPC with 7.5-RSMP using mineral salts. $CaCl_2$ added RMPC sample could not be UHT processed due to its low heat stability. Addition of NaCl to RMPC did not adversely affect their UHT stability (UHT run-time >120 min). KCl caused a decrease in UHT stability (run-time 15 min) and a drop in overall heat transfer co-efficient (OHTC) values. High apparent viscosity and formation of larger particles were observed in KCl added RMPC as compared to control RMPC. Overall mineral balance with reduced amount of minerals per protein unit were found to be responsible for high heat stability for RMPC compared to RSMP.

Effect of relative concentrations of caseins and whey proteins on UHT stability of high protein dispersions was also studied. RMPC, reconstituted whey protein concentrate (RWPC) and samples with various casein to whey protein ratios (C:W) (80:20 to 40:60) were UHT processed. A 2% protein RWPC showed severe fouling suggesting its poor UHT stability. Inclusion of caseins caused stabilization of whey proteins to UHT processing and 10% protein C:W-50:50 was successfully processed for >120 min suggesting the chaperone protein like activity of caseins protecting whey

proteins against thermal stresses. Further increase in whey proteins proportion caused a drop in run-times (<120 min) and OHTC, corresponding to an increase in particle size and apparent viscosity. Presence of higher amounts of casein in the serum phase of samples caused the formation of smaller protein aggregates (D(4,3) was 0.23 and 0.16 μm for supernatants of C:W-40:60 and RMPC, respectively) after heating.

The knowledge gained from above mentioned studies was applied to formulate an RTD beverage. The effect of two 10% milk protein formulation bases: i) RMPC base (RMPC-Choco) and ii) RMPC and RWPC base (C:W-Choco) and different κ-carrageenan concentrations (0, 0.01, 0.03 and 0.05%) on UHT stability of chocolate flavoured high protein beverages were studied. Samples without the addition of κ-carrageenan showed severe fouling and very short UHT run-times due to settlement of cocoa powder particles in the processing line. It was found that addition of 0.03 and 0.01% κ-carrageenan to RMPC-Choco and C:W-Choco, respectively, made them UHT stable (UHT run-times >120 min) due to formation of a weak gel by milk protein- κ-carrageenan interactions entrapping cocoa particles. High UHT stability of C:W-Choco at low levels of κ-carrageenan was linked to additional gelation of higher amounts of whey proteins present in the formulation. However, a loss of fluidity in C:W-Choco samples with 0.03 and 0.05% κ-carrageenan was observed. During a sensory evaluation no significant difference for overall preference among three selected samples (RMPC-Choco with 0.03 and 0.05% κ-carrageenan and C:W-Choco with 0.01% κ-carrageenan) was found.

The objective of the last study in this thesis was to investigate the effect of the addition of soy protein hydrolysates on UHT stability of high protein milk beverages. UHT stability of 8% protein RMPC (8-RMPC), 8% protein soy protein hydrolysate (8-RSPH) and 8-RMPC with added 1, 2 and 3% protein RSPH (e.g. 8-RMPC+1-RSPH) was studied. Both 8-RMPC and 8-RSPH had high UHT stability (UHT run-time >120 min). Inclusion of 1-RSPH in 8-RMPC did not affect UHT run-time (>120 min) and OHTC. Significant drops in OHTC was observed in 8-RMPC+2-RSPH without reducing the UHT run-time (>120 min). 8-RMPC+3-RSPH showed markedly reduced UHT stability (UHT run-time 61 min and low OHTC values) due to the formation of larger protein aggregates and increase in apparent viscosity.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications included in this thesis

1. **Singh, J.**, Prakash, S., Bhandari, B., & Bansal, N. (2019). Comparison of ultra-high temperature (UHT) stability of high protein milk dispersions prepared from milk protein concentrate (MPC) and conventional low heat skimmed milk powder (SMP). Journal of Food Engineering, 246, 86-94.

2. **Singh, J.**, Prakash, S., Bhandari, B., & Bansal, N. (2019). Ultra high temperature (UHT) stability of casein-whey protein mixtures at high protein content: Heat induced protein interactions. Food Research International, 116, 103-113.

Submitted manuscripts included in this thesis

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Contributions by others to the thesis

Senior lecturer Dr Nidhi Bansal, Professor Bhesh Bhandari and lecturer Dr Sangeeta Prakash have contributed to conceptualize and design of the project and helped in critical revision of the thesis content. Dr Zhihua pang helped in sourcing soy protein hydrolysate samples to conduct experiments for Chapter 7. Ms Agathe Dean helped in heat coagulation time data collection for Chapter 4.

Statement of parts of the thesis submitted to qualify for the award of another degree

No works submitted towards another degree have been included in this thesis.

Research involving human or animal subjects

Ethics approval for sensory evaluation involving human volunteers was obtained from The University of Queensland (Approval number 2019001010).

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Keywords

Ultra-high temperature processing, high protein beverages, thermal stability, fouling, caseins, whey proteins

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

AOAC	Association of Official Agricultural Chemists
BSA	Bovine serum albumin
C:W	Casein to whey protein ratio
Choco	Chocolate
HCT	Heat coagulation time
Ig	Immunoglobulins
Lf	Lactoferrin
Lp	Lactoperoxidase
MPC	Milk protein concentrate
MPI	Milk protein isolate
NR SDS-PAGE	Non-reducing SDS-PAGE
OHTC	Overall heat transfer coefficient
PSD	Particle size distribution
R SDS-PAGE	Reducing SDS-PAGE
RMPC	Reconstituted milk protein concentrate
RSMP	Reconstituted skim milk powder
RSPH	Reconstituted soy protein hydrolysate
RSPI	Reconstituted soy protein isolate
RTD	Ready to drink
RWPC	Reconstituted whey protein concentrated
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
SMP	Skim milk powder
SMUF	Simulated milk ultrafiltrate
SPH	Soy protein hydrolysate
SPI	Soy protein isolate
UHT	Ultra-high temperature
WPC	Whey protein concentrate
WPI	Whey protein isolate
α-la	Alpha-lactalbumin
β-lg	Beta-lactoglobulin

CHAPTER 1: INTRODUCTION

1.1 Background

Shelf stable ready to drink (RTD) high protein beverages are protein supplement drinks prepared from milk protein and plant protein sources (Agarwal, Beausire, Patel, & Patel, 2015; Childs, Yates, & Drake, 2007; Durand, Franks, & Hosken, 2003; Kelly, Woonton, Smithers, & Paquin, 2009; Walters, 2004). These products are popular among dieters, athletes and health conscious consumers due to their high protein content in a convenient package, which provide consumers much of their daily intake of the protein without consuming large volumes of food. RTD high protein drinks are available in the market as commercially sterilized products in various flavours and appearances to attract potential consumers. Ultra high temperature (UHT) treatment is a commonly used method for manufacturing commercially sterilized dairy products. There is a major challenge associated with UHT treatment of milk products, which is the fouling of heat transfer surfaces by the gradual build-up of heat sensitive constituents of process fluid on heat transfer surfaces to form a deposit layer, which limits run time of UHT operation (Sadeghinezhad et al., 2013).

Milk protein concentrate and isolate (MPC and MPI), whey protein concentrate and isolate (WPC and WPI), caseinates and other concentrated milk products are used as ingredients in the formulation of high protein beverages. Manufacturers usually combine two or more protein powders to obtain the desired product. Due to that the composition and food system of high protein beverages can be very different from milk, but the problems associated with the processing of UHT milk are also encountered in these products (Deeth, 2010).

The RTD high protein beverages are required to contain high protein levels without compromising their thermal stability and product quality. The milk proteins, particularly the whey proteins are not very heat stable at high temperatures, especially when present in the system at high concentrations (Fickak, Al-Raisi, & Chen, 2011; Prakash, Datta, & Deeth, 2005). Hence, there is a need to gain a better understanding of the behaviour of concentrated milk protein dispersions during UHT treatment of these products.

This PhD project is being undertaken to realise the following objectives based on hypotheses described below:

1.2 Research aims and objectives

This project was aimed to study the stability of high milk protein dispersions at UHT temperatures, investigated through their fouling behaviour, milk protein interactions and physicochemical changes during UHT processing.

This broad aim was achieved through the following objectives:

- To study the UHT fouling behaviour and physicochemical properties of milk protein dispersions prepared from different milk protein ingredients in order to achieve high protein content UHT stable product and to establish baseline total solids and protein concentrations for further studies.
- To compare the effect of compositional differences of milk protein ingredients on UHT stability of milk protein dispersions prepared from them.
- To utilise the knowledge gained from UHT behaviour of different milk protein ingredients to develop a UHT stable milk protein dispersion with high whey protein content.
- To study the UHT stability of high milk protein beverage bases prepared with other added ingredients such as plant protein sources, stabilisers and flavouring ingredients.

1.3 Hypotheses

- The UHT stability of high milk protein beverages is a function of their protein content and physicochemical properties of milk protein ingredients used to prepare them. The most important physicochemical property of high milk protein dispersions influencing their UHT stability is denaturation and aggregation of milk proteins.
- Presence of high concentration of milk proteins in the system results in increased protein interactions at elevated temperatures.
- The difference in physicochemical properties of milk protein ingredients is due to their compositional differences such as milk protein type, the relative abundance of different types of milk proteins and mineral balance.
- These compositional differences among milk powders and high concentration of milk proteins in the system govern milk protein interactions and deposit formation during UHT processing of high milk protein dispersions.
- Two or more milk powders can be mixed together to achieve a high protein beverage base which does not foul heat exchange surfaces readily, e.g. a high casein content milk powder can be mixed with a whey protein concentrate powder to achieve UHT stable high whey protein dispersion, exploiting the molecular chaperone like activity of caseins.
- Addition of other ingredients such as plant protein ingredient, stabilisers, flavouring ingredient to a milk protein beverage mix affects its UHT stability.

1.4 Expected outcomes

The outcomes of this research project could help to improve our understanding of the physicochemical basis of improved heat stability of a milk protein product processed at UHT conditions, formulated using carefully selected milk protein ingredients. The knowledge acquired through this work will potentially assist in formulating UHT stable high milk protein mixes used for sports, elderly, medical and general nutrition products.

1.5 Outline of the dissertation

This thesis consist of 8 chapters, which start with a general introduction (Chapter 1) followed by a literature review (Chapter 2). The research undertaken in this project is presented in five consecutive chapters (Chapters 3 to 7) in a format of journal manuscripts. Chapter 8 provides general conclusions and recommendations from the research.

Chapter 1 describes a background understanding related to the UHT processed high protein beverages and the problem of fouling during UHT processing of milk products.

Chapter 2 presents a review of literature related to the milk protein composition, UHT processing technology, fouling of milk products during UHT processing, product and process factors affecting fouling and strategies to minimize fouling. A special focus is given to the literature review of the role of milk proteins and minerals in fouling.

Chapter 3 compares the UHT fouling behaviour of reconstituted milk protein concentrate (RMPC) with reconstituted conventional low heat skimmed milk powder (RSMP). This chapter also deals with the effect of increased protein content on the extent of deposit formation during UHT processing of high protein dispersions.

Chapter 4 provides more insights into the reasons behind higher heat stability of RMPC as compared to RSMP. The role of minerals of UHT stability of MPC is studied in this chapter.

Chapter 5 describes the effect of chaperon activity of caseins on whey protein aggregation in order to obtain a UHT stable high milk protein beverage base containing high amounts of whey proteins.

Chapter 6 describes the effect of the addition of cocoa powder and κ-carrageenan on UHT stability of high protein beverage mixes. The sediment formation, rheology, tribology and other physical properties related to a beverage mix are studied.

Chapter 7 discusses the influence of incorporating soy protein derived small molecular peptides to MPC based high protein beverages. The changes in fouling behaviour and physicochemical properties of MPC after the addition of soy protein hydrolysates are studied.

Chapter 8 provides overall conclusions and recommendations for further studies on this research work.

CHAPTER 2: LITERATURE REVIEW

2.1 Milk proteins

Milk proteins are classified into two broad groups: caseins and whey proteins (Raikos, 2010). Apart from these two groups there are other proteins present in milk in minor quantities. Caseins can be precipitated from milk at pH 4.6 and 20 ^{0}C and the whey proteins remain soluble in the supernatant (Farrell Jr et al., 2004). Milk proteins have various nutritional and physiological benefits. The nutritional, physical and chemical properties of milk proteins are exploited through various processing techniques during the manufacturing of dairy products. The thermal processing of milk is affected more by the concentration and properties of milk proteins than by any other milk constituent (Sikand, Tong, & Walker, 2010). Milk proteins are one of the most extensively studied food protein system due to their importance in the human diet and this knowledge is also crucial in designing new products and optimizing the processes involved in dairy products development.

Table 2.1: Proteins in cow's milk: approximate composition.

Protein	g/kg of milk	g/100g of total protein
Total Casein	26	78.3
α_{S1}-Casein	10.7	32
α_{S2}-Casein	2.8	8.4
β-Casein	8.6	26
κ-Casein	3.1	9.3
γ-Casein	0.8	2.4
Whey proteins	6.3	19
β-Lactoglobulin	3.2	9.8
α-Lactalbumin	1.2	3.7
Blood serum albumin	0.4	1.2
Protease and peptone	0.8	2.4
Immunoglobulins	0.8	2.4
Other Proteins (Membrane proteins, enzymes etc.)	0.9	2.7

Adapted from (Walstra, Wouters, & Geurts, 2005)

2.1.1 Caseins

Caseins are phosphoproteins, the largest group (~80%) of proteins in cow's milk and are present in milk in the form of large spherical colloidal aggregates called casein micelles, which are made up of individual casein molecules to form a higher order stable aggregated structure (Fox & Brodkorb,

2008). Four major types of casein proteins present in milk are: α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein. These four types of caseins have different structure and amino acid composition, and hence have different physicochemical properties associated with them (Holt, Carver, Ecroyd, & Thorn, 2013; Huppertz, 2013; O'Mahony & Fox, 2013).

The caseins contain substituted phosphate on their serine side chains. The α_{s1}-casein, α_{s2}-casein and β-casein are highly phosphorylated (Phadungath, 2005). Attachment of calcium to serine phosphate residues on casein molecules by electrostatic interactions can lead to precipitation of caseins at typical milk calcium concentrations, but κ-casein, which stabilizes the casein micelle is insensitive to calcium and does not precipitate in excess calcium concentrations (Huppertz, 2013). The four different types of casein molecules form casein micelles via non-covalent interactions with each other and with colloidal calcium phosphate (CCP) (Dalgleish & Corredig, 2012).

The association of CCP with caseins serves a biological function by enabling the milk to carry a high concentration of calcium phosphate in soluble form. This makes caseins the primary source of protein, calcium and phosphate for mammalian neonates (McMahon & Brown, 1984; O'Mahony & Fox, 2013). Three types of casein micelle models: coat-core model, sub-micelle (subunit) model and internal structure model have been proposed by researchers (Phadungath, 2005). Caseins have a low content of disulphide bonds, which in turn responsible for the low secondary and tertiary structure. This makes it very resistant to thermal denaturation. However α_{s2}-casein and κ-casein contain Cysteine, which makes them susceptible to associate with whey proteins during thermal treatment (Kinsella & Morr, 1984; O'Mahony & Fox, 2013; Wong, Camirand, Pavlath, Parris, & Friedman, 1996). The C-terminal of κ-casein is very hydrophilic and possesses high net negative charges and protrudes outside of the casein micelle. This forms a hairy layer on the surface of casein micelle which hinders further growth of micelle and stabilize it due to electrostatic and steric stabilisation (Horne, 2011; Phadungath, 2005).

2.1.2 Whey proteins

Whey proteins are present in the milk dissolved in the serum; these remain in the supernatant of milk after precipitation of caseins (Surroca, Haverkamp, & Heck, 2002). Whey proteins are highly structured globular proteins showing tertiary and quaternary structure. Whey proteins constitute around 20% of the total cow milk proteins. The heterogeneity among whey proteins is large as compared to caseins (Walstra et al., 2005). Only the whey protein fractions: β-Lactoglobulin (β-lg) and α-Lactoalbumain (α-la), which account for ~70% of total whey proteins in bovine milk and play a major role during thermal treatment of milk are discussed in this section (De La Fuente, Singh, & Hemar, 2002; Schokker, Singh, & Creamer, 2000).

β-Lactoglobulin is the major whey protein representing nearly half of the total whey proteins (Table 2.1). It contains two disulphide linkages and a very reactive free sulfhydryl group (Considine, Patel, Anema, Singh, & Creamer, 2007). Usually two β-lg molecules are tightly bound to each other by hydrophobic bonds and are found in the milk as dimers (Walstra et al., 2005). These dimers dissociate and β-lg undergoes structural changes during heating over 60 ^{0}C. Fouling of heat exchanging surfaces during Ultra high temperature (UHT) treatment is largely dependent on the denaturation and aggregation of β-lg and its association with caseins and α-la molecules to produce larger insoluble aggregates (Lalande & Tissier, 1985).

α-Lactalbumin is a compact globular protein comprising around 20% of the total whey proteins in cows' milk (Table 2.1). It contains no free sulfhydryl groups but it includes four intramolecular disulphide groups, which makes its structure stable (O'Mahony & Fox, 2013). α-la binds calcium strongly, which stabilizes its conformation, however it can partially undergo structural changes when calcium is removed or it loses its bound calcium at pH near 4 (Walstra et al., 2005).

2.2 Ready to drink (RTD) high protein milk beverages

Health and sports products manufacturers are constantly looking into developing new products with better health benefits and high nutritional value in more convenient forms for their potential consumers. Increased consumer awareness towards the benefits of protein in the diet and its positive effects on weight loss and muscle building has surged the demand for high protein weight loss diets (Friedman, 2004). There are various formats of high protein processed foods available in the market, but RTD high protein beverages are a notable departure from typical powder type sports supplements specifically targeted at body builders and sports persons (Baxter, Dimler, & Rangavajala, 2011). Due to their convenience, high protein beverages are also appealing to health conscious ordinary consumers. RTD high protein beverages based on milk proteins are processed liquid food products. These drinks are available in the market in various flavours and serving sizes.

Manufactures combine high protein dairy powders along with other ingredients to obtain a ready to drink product with desired protein, fat and carbohydrate content, amino acid profile and sensory attributes. (Baxter et al., 2011; Jelen, 2011; Lönnerdal & Hernell, 1998). The development of a UHT treated milk protein beverage, from formulation to a finished product, involves many steps and it is a very complex process, including the selection of ingredients and their processing through different processing regimes. Different formulations can be prepared to suit ordinary consumers, sportspersons, patients, elderly and infant formulas. Every ingredient has a particular role for providing nutrition to targeted consumers. Protein provides amino acids for muscle maintenance, carbohydrates and fat provide energy, water provides hydration and minerals and salts provide

electrolytes to the body (Whitney, Rolfes, Crowe, Cameron-Smith, & Walsh, 2011). Apart from nutrition the other basis of ingredient selection is their functional and sensory properties. This is an area where sweeteners, flavouring agents and stabilisers find their application (Boomgaard, van Vliet, & van Hooydonk, 1987). Common flavouring agents used in neutral pH beverages are vanilla, chocolate and orange cream (Beecher, Drake, Luck, & Foegeding, 2008b). Stabilisers such as gums (carrageenan, carboxymethyl cellulose etc), phosphates and citrates are used to stabilise proteins in solution, improve texture and storage stability of product (Datta & Deeth, 2001; Phillips & Williams, 2000). Plant protein ingredients such as soy protein isolate and pea protein isolates are also included in some high protein beverage formulation (Lan, Chen, & Rao, 2018). However milk powders, casein and whey protein concentrates and isolates form the base of milk protein based formulations. These powders are widely produced commercially and are used in the formulation of many food products.

2.3 Milk protein ingredients

The concentrated powder forms of milk solids are a rich source of high quality milk proteins. Different types of protein rich powders are commercially produced from bovine milk using various unit operations. Calcium, potassium and sodium caseinates, whey protein concentrates (WPC) and isolates (WPI), skimmed milk powder (SMP), whole milk powder (WMP), milk protein concentrates (MPC) and isolates (MPI) and micellar casein concentrate (MCC) serve as major milk protein ingredients for various milk protein based products. These milk powders differ from each other in their protein content, type and the relative ratio of individual milk proteins and the method of accumulation and separation of milk proteins (Deeth & Hartanto, 2009). These variations in the products also give them different physicochemical and functional properties, which are important when the powders are intended for use as ingredients for milk protein based products. Table 2.2 shows the proximate composition of milk and some dried milk products.

A brief description of some of the common milk protein ingredients used in food product development is given below.

Table 2.2: Proximate composition (g/100g) of milk and dried milk products.

Product	Water	Fat	Protein	Carbohydrates	Ash/minerals
			(g/100g)		
Liquid Milks					
Whole Milk	87	3.7	3.3	4.8	0.7
Skimmed Milk	90	<0.1	3.4	4.9	0.75
Milk Powders					
WMP	2-4	25-28	25-27	37-38	6-7
SMP	3-5	0.7-1.3	35-37	49-52	7.5-8.0
Milk and whey protein powders					
MPC 42	3.5	1.0	42	46	7.5
MPC 70	4.2	1.4	70	16.2	8.2
MPC 75	5.0	1.5	75	10.9	7.6
MPC 80	3.9	1.8	80	4.1	7.4
MPC 85	4.9	1.6	85	1.0	7.1
Caseinate (calcium caseinate, potassium caseinate, sodium caseinate)	3-5	0.9-1.5	89-95	0.2	3.3-5
Low protein WPC	4.6	2-4	34-36	44-53	7-8
Medium protein WPC	4.3	5	53	35	7
High protein WPC	3-4	4-6	59-65	21-22	3.5-4
Very high protein WPC	4-5	03-7.0	72-81	2-13	2.5-6.5
WPI	2.5-6	0.1-0.7	89-93	0.1-0.8	1.4-3.8
Fractionated whey proteins					
α-fraction	4.5	1.0	81.5	7	3.4
β-fraction	4.5	0.4	87	0.5	3.0

Adapted from (Deeth & Hartanto, 2009)

2.3.1 Whole milk powder (WMP) and Skim milk powder (SMP)

WMP and SMP are very popular dairy based ingredients and are used in a wide variety of foods because of their functional properties. WMP is produced by removing water, whereas SMP is produced by removing water and milk fat from liquid milk (Sharma, Jana, & Chavan, 2012). SMP can be generally classified according to the severity of preheat treatment used on skim milk before concentration and dehydration. There are three main classifications: low heat SMP (LHSMP), medium heat SMP (MHSMP) and high heat SMP (HHSMP) (McKenna & Singh, 1991). Whey protein nitrogen index (WPNI) is a measure for undenatured whey proteins in SMP and helps to classify SMPs. LHSMP, MHSMP and HHSMP contain $\geq$6.0, 1.51-5.99 and $\leq$1.50 mg of undenatured whey protein per g of powder, respectively (Sharma et al., 2012). Poor storage stability, off flavour development and inferior dispersibility limits the value of WMP as an ingredient in beverage products. By contrast LHSMP are more popularly used in milk protein based beverages. While SMP with low WPNI are used in confectionary and bakery products (Kinsella & Morr, 1984).

2.3.2 Milk protein concentrates (MPC) and isolates (MPI)

Milk proteins concentrates (MPC) are manufactured from skim milk by ultrafiltration and diafiltration process, followed by spray drying the retentate (Havea, 2006). The filtration process also partially removes lactose and mineral salts from the milk. The solid concentration in retentate can be further increased by membrane filtration process for the production of MPI with a protein content of around 90% (Huffman & James Harper, 1999). These powders are complete dairy proteins, containing casein and whey proteins in their original proportions found in milk and milk proteins also are in their native state. Much of the caseins in concentrates are in micellar form and whey proteins are largely undenatured (Agarwal et al., 2015; Augustin, Oliver, & Hemar, 2011). The protein content of MPC varies from 42 to 85%, which is indicated by the number following MPC (Deeth & Hartanto, 2009). MPCs find application in the manufacture of food emulsions, cheese, yogurt, ice cream, health related products and various other dairy products (Kelly, 2011). MPCs/MPIs have a very mild, milky flavour, which does not interfere with flavouring agents used in the formulation of beverages (Chandan, 2015).

2.3.3 Whey protein concentrates (WPC) and isolates (WPI)

Whey, once considered as a waste product of cheese making process, is now considered as an excellent source of essential amino acids (Smithers, 2008). WPC and WPI are produced from liquid whey by membrane filtration techniques followed by drying to make a protein rich powder. Manufacturing of whey protein concentrates requires removal of non-protein components i.e. water,

lactose, ash and lipid from the liquid whey, which gives a final product containing a varying percentage of protein content and other constituents (Huffman & James Harper, 1999). WPCs have protein content from 34-35% to around 80%, while WPIs are mostly proteins and contain 90% or more of it (Kehoe & Foegeding, 2011). WPIs are more expensive than WPCs due to high cost of production and are used in high-end products (Huffman & James Harper, 1999; Morr & Ha, 1993).

2.3.4 Caseinates

Water soluble commercial caseinates are obtained by solubilizing acid casein slurry with alkali and the solution is then dried to obtain a powder (Huffman & James Harper, 1999). Calcium, potassium, ammonium and sodium hydroxide treated acid caseins produce calcium, potassium, ammonium and sodium caseinate, respectively. The pH of caseinates ranges from 6.5 to 7.0 (Augustin et al., 2011). Sodium and calcium derivatives are the most common. All the caseinates have similar physical properties. They produce straw coloured, translucent, viscous solution in water, however calcium caseinate solution are turbid or milky due to its tendency to form aggregates in water. Sodium caseinate is highly soluble in water, highly heat resistant and have excellent water binding properties (Augustin et al., 2011). Caseinates have little similarity to native casein micelles because of the acid and alkaline treatments disrupt the native structure of the casein micelles (Kinsella & Morr, 1984).

2.3.5 Micellar casein concentrate (MCC)

MCC is produced from skim milk by microfiltration process, which separates whey proteins from casein micelles. The separation is based on the large size difference between casein micelles and whey proteins, caseins are approximately 10 to 100 folds larger in diameter than whey proteins (Hurt, Zulewska, Newbold, & Barbano, 2010). The concentrate can be dried to manufacture MCC powder. Whey proteins separated by microfiltration can also be further converted to powder forms and these are valuable ingredients to develop food products (Beckman, Zulewska, Newbold, & Barbano, 2010; Hurt et al., 2010). As compared to traditional methods of casein separation i.e. acidification or coagulation, microfiltration process does not destroy the native structure of casein micelles (Al-Akoum, Ding, & Jaffrin, 2002; Espina, Jaffrin, Frappart, & Ding, 2008). MCC are used in cheese production by mixing it with cream. These are also used for standardisation of milk and as an ingredient in milk protein products (Espina et al., 2008).

2.4 UHT processing of dairy products

Several heat treatment techniques are used to ensure an extended shelf life of milk and milk products; UHT method is the severest of all. UHT is a continuous process, which renders the product commercially sterilized. Compared to the retort sterilization process, there are less colour and flavour

changes and minimal losses of nutrients during UHT due to very short holding time and faster heat transfer (Burton, 1994; Deeth, 2010).

The UHT processing technology is a method of sterilizing milk and milk products in a continuous process at temperatures higher than 130^0C (usually 138-145 ^{0}C), which requires holding time of 1-10 s (usually 3-5 s) (Deeth & Datta, 2011), followed by rapid cooling to avoid further thermal damage to the product. The product is then aseptically packaged. Different time temperature combinations of UHT processing ensure a commercially sterile and shelf stable product for longer storage periods. The time temperature combinations for UHT treatment are based on achieving a sterile product while ensuring minimal heat damage to it (Deeth & Datta, 2011).

UHT milk has a long shelf life without the need for refrigeration, which reduces costs during storage and transportation and also makes it possible to deliver the product over long distances and to the remote areas (Gedam, Prasad, Vijay, & Chanda, 2007). However, there can be undesirable interactions among milk proteins and other milk components during UHT processing, which can destabilise the protein structures leading to processing issues. Issues of heat stability of milk proteins and the formation of fouling deposits on heated surfaces during UHT are ongoing problems in the dairy industry (Burton, 1994). In addition to that there is a loss of stability in UHT milk during storage, which is a result of the heat induced changes to the milk constituents and the reactions that occur during processing (Datta & Deeth, 2001; Manji & Kakuda, 1988). These reactions can continue during storage but at a slow speed due to low storage temperatures. Age gelation and sedimentation are the other two major problems associated with UHT milk products (Burton, 1994; Datta & Deeth, 2007).

Some formats of high protein beverages are required to be commercially sterilized to make them shelf stable for a longer storage period. Therefore, the problems associated with UHT processing of milk products and subsequent storage of UHT milk products are also encountered in high milk protein beverages formulated from milk protein powders. Milk proteins are the main ingredients of these beverages and owing to their sensitivity to high heat considerable research has been conducted on the stability of milk proteins during processing.

2.5 General aspects of fouling of heat exchanging surfaces

Fouling is the formation of layers of deposits on the heat exchanging surfaces that may negatively impact the heat transfer to product from the heating medium and mass flow rate of the product in the heat exchangers, which can lead to inadequate processing of the product (Bansal & Chen, 2006). Fouling limits run time of manufacturing plants because frequent shut downs are necessary to clean

the deposits from the processing equipment (Bansal & Chen, 2006). This costs manufacturers in the form of lost hours of operations and the use of chemicals and energy during cleaning (Lyster, 1965; Visser & Jeurnink, 1997). There are also concerns about safe disposal of cleaning chemicals which are harmful to the environment. Moreover contamination of the product by dislodged fouling deposits from the surfaces and growth of microflora on the fouling deposits can deteriorate product quality (Beuf et al., 2003; Goode, Asteriadou, Robbins, & Fryer, 2013). Fouling is a major cause of concern in the dairy industry, since most of the milk products undergo some kind of heat treatment of varying time temperature combinations during their manufacturing process. Milk is a complex biological fluid containing proteins, fat, carbohydrates and minerals (Deeth & Hartanto, 2009; Walstra et al., 2005), which makes the study of milk fouling complicated.

Researchers has classified milk fouling into Type A and Type B based on their temperature of formation and composition of the deposits (Bansal & Chen, 2006; Burton, 1968; Changani, Belmar-Beiny, & Fryer, 1997; Hooper, Paterson, & Wilson, 2006; Lalande, Tissier, & Corrieu, 1984; Xin, 2003). Composition of deposits is affected by type and composition of dairy product being processed (e.g. milk, high protein drinks), time-temperature combinations (e.g. pasteurization, sterilization) and type of processing technique being used for processing (e.g. direct or indirect UHT treatment) (Bansal & Chen, 2006). Type A deposits form in the temperature range of 75-110 ^{0}C and these are soft and voluminous proteinaceous deposits, usually white or cream in colour (Lalande et al., 1984). At the lower end of the temperature range (typical UHT preheating temperatures), Type A deposits mainly consist of denatured β-Lg, these shift to high casein content at high temperatures (Tissier, 1984). Brown or grey coloured Type B deposits, on the other hand, occur at temperatures above 110 ^{0}C and are a cause of fouling in sterilization section in UHT plants. Type B deposits are largely minerals (70 to 80%), with protein and fat being minor constituents (Lalande et al., 1984).

The build-up of fouling layer is a transient process, which starts with an induction phase followed by a fouling phase (Belmar-Beiny & Fryer, 1993; Paterson & Fryer, 1988; Visser & Jeurnink, 1997). Modification of heat exchanging surface occurs during the induction phase, due to initial interactions with heated bulk fluid. This occurs mainly by physicochemical changes in milk constituents in heated bulk fluid and conversion of some of its constituents in a form that is capable of sticking to the heat transfer surfaces (Burton, 1994; Morr, 1985). However Jeurnink, Verheul, Stuart, and de Kruif (1996) and Arnebrant, Barton, and Nylander (1987) reported that a solution containing whey proteins can adsorb at stainless steel surfaces even at room temperatures forming a monolayer of proteins on the surface.

When the temperature of the bulk fluid increases to denaturation temperature of whey proteins, aggregation begins and further layers of deposits start to build up (Burton, 1994). At temperatures above 60^0C whey proteins, mainly β-Lg starts to denature, exposing their sulfhydryl groups, which start binding with other whey proteins and caseins via thiol sulfhydryl interchange reactions forming larger insoluble aggregates in bulk fluid (Jeurnink, Walstra, & De Kruif, 1996). There is a direct relationship between the amount of denatured β-Lg molecules and the rate of fouling (Itoh et al., 1995; Skudder, Thomas, Pavey, & Perkin, 1981). In addition to that, solubility of calcium phosphate decreases at high temperatures (Fox, 1981; Lalande, Rene, & Tissier, 1989; Lalande et al., 1984; Visser & Jeurnink, 1997). Precipitation of minerals and attachment of protein aggregates or activated whey proteins to the heat transfer surfaces causes formation of an initial deposit layer (contributes to Type B and Type A deposits, respectively). This initial deposit layer forms the basis of deposition of further material that leads to start a fouling phase (Belmar-Beiny & Fryer, 1993). Activated β-Lg with its exposed sulfhydryl group can attach to this initial proteinaceous layer via formation of disulphide bonds, which depends on the orientation of exposed sulfhydryl group on the activated β-Lg in bulk fluid and the protein molecules already deposited on the surface (Jeurnink, Verheul, et al., 1996). The deposits layer then keeps on growing in thickness with time, slightly compensated by possible removal of deposits via shearing caused by the fluid flowing over fouled material (Foster & Green, 1990).

2.6 Role of milk proteins and minerals in UHT fouling and heat stability of milk products

Denaturation of proteins is a process where the native structure of proteins molecules gets unfolded and loses its native conformation and proceed to a disordered state, which exposes originally buried hydrophobic amino acid side chains and other reactive groups (Walstra et al., 2005). Denaturation does not change the amino acid sequence of the proteins but it disrupts the stability of proteins in the system and may lead to subsequent aggregation of unfolded proteins. When milk protein system is heat treated, a complex mixture of native whey proteins, whey protein aggregates and whey protein-casein complexes is formed (Wijayanti, Bansal, & Deeth, 2014). Since milk protein system is a complex system, the heat denaturation and aggregation behaviour of purified individual proteins cannot be directly compared to their behaviour in product during thermal processing. The heat stability of milk proteins and their susceptibility to denaturation and aggregation and final composition of this mixture depends on the temperature and time of heating, pH, relative abundance of other proteins and salts in the food system (Crowley, Dowling, Caldeo, Kelly, & O'Mahony, 2016b; Crowley, Megemont, et al., 2014; Muir, 1985; Singh, 2004).

In the case of UHT processing of milk and milk protein products, the dominant protein found in fouling is β-Lg, which is a major constituent of Type A fouling (Tissier, 1984). In general, when heated, β-Lg dimers dissociate into monomers, which is a reversible process. These monomers undergo partial unfolding on further heating. Temperatures above 70^0C lead to irreversible conformational changes in β-Lg. Denaturation of β-Lg exposes its reactive sulfhydryl group, which can further initiate sulfhydryl disulphide group exchange interactions. The reactive sulfhydryl group reacts with a disulphide bond and forms a new reactive sulfhydryl group, which further continues the reaction (Muir, 1985; Wijayanti et al., 2014). The other major whey protein α-La only contains disulphide bonds and due to the absence of free sulfhydryl groups it is able to renature again (De Wit & Klarenbeek, 1984). If heated alone below 95^0C, more than 90% of α-La can return to its original state, but the ability of α-La to renature decreases with increased temperature. On the other hand, when heated in the presence of exposed sulfhydryl groups of β-Lg, α-La irreversibly denatures due to sulfhydryl disulphide group exchange reactions between these two proteins (Calvo, Leaver, & Banks, 1993; Chaplin & Lyster, 1986). Crowley et al. (2016b) showed that increased concentration of β-Lg in the system adversely affects the heat stability of protein dispersions containing β-Lg and α-La. These intramolecular and intermolecular interactions lead to the formation of larger whey protein aggregates via a polymerization like process and the process is terminated when sulfhydryl groups on reactive intermediates are consumed to form larger stable aggregates (Vasbinder & De Kruif, 2003). The formation of whey protein aggregates is an entirely irreversible process and the resulting aggregates are insoluble in the solution (Wijayanti et al., 2014). This can lead to settling out (sedimentation) of whey proteins not only during UHT processing but also during storage of processed milk products, limiting the quality of the product (McKenna & Singh, 1991). However sedimentation depends strongly on the particle size and on the difference in mass density between particles and milk serum (Datta, Elliott, Perkins, & Deeth, 2002; Koffi, Shewfelt, & Wicker, 2005; McKenna & Singh, 1991; Nieuwenhuijse & van Boekel, 2003).

Caseins molecules in casein micelles and milk serum are present as random coils and these proteins contain little ordered structure to be disrupted by high heat, therefore caseins are more stable to high temperatures as compared to whey proteins (Fox, 1981). The other factor responsible for casein micelle stability is the steric stabilisation provided by an outer κ-casein layer that prevent flocculation caused by Van der Waals interactions between micelles (De Kruif, 1999).

Heat treatment of milk at typical UHT temperatures causes depletion of CCP, decrease in pH, increase in average casein micelle size, dissociation of caseins and coagulation of micelles (Singh, 2004). The change in surface charge of casein micelles and the change in the state of CCP during heating are

responsible for reduced stability and dissociation of casein micelles (Holt, Davies, & Law, 1986). The casein micelles are negatively charged at normal pH of the milk. The net amount of charge is dependent upon the amount of calcium bound to the casein micelle and free Ca^{2+} present in the serum phase of the milk. If the pH of milk is decreased, Ca^{2+} are released into the serum from the interior of the micelles through the dissolution of CCP and thus there is an increase in free Ca^{2+} in the serum, leading to micelle destabilisation (Dalgleish & Corredig, 2012; De Kruif, 1999; Horne, 1998; Singh, 2004). Furthermore, as mentioned earlier in section 2.5, calcium phosphate has reverse solubility with increasing temperature, causing CCP to precipitate from casein micelles. This destabilises casein micelle structure and leads to coagulation of micelles contributing to mineral and protein (Type-B) fouling of heating surfaces (Burdett, 1974; Fox, 1981; Lalande et al., 1989; Lalande et al., 1984).

There is an increase in casein dissociation from casein micelles with an increase in pH and temperature of milk which follows a trend of κ-casein > β-casein > α_s-caseins when comparing individual dissociation of caseins from micelles. The caseins dissociate from micelle rapidly during the initial stages of heating and there is a little change with increased heating time. However, the mechanism of dissociation is unknown, but as discussed above, modifications of casein micelles via changes in CCP, hydrophobic interactions between casein micelles due to increase in temperature and pH may be responsible for their dissociation (Anema, 1998; Anema & Klostermeyer, 1997). Casein micelles without hairy brush of a κ-casein are less stable and coagulation of casein micelles can occur due to loss of the colloidal stability, as the steric stabilisation provide by κ-casein is not available (Dalgleish & Corredig, 2012).

Whey proteins interact with κ-casein present on the surface of casein micelles and in the serum through hydrophobic and sulfhydryl disulphide group exchange interactions, which leads to increase in size and other physicochemical changes in properties of the casein micelles (Anema & Li, 2003a; Hill, 1989; Jang & Swaisgood, 1990; Ono, Yoshida, Tanaami, & Ohkosi, 1999; Sawyer, 1968). The method of heat treatment affects the rate and degree of interactions between whey proteins and casein micelles (Corredig & Dalgleish, 1996). Slow heating of milk using indirect method caused association of almost 80% of the denatured β-lg with casein micelles (Smits & Van Brouwershaven, 1980). In comparison, when milk was heated rapidly using the direct method of heating, only half of the whey proteins bound with casein micelles (Oldfield, Singh, Taylor, & Pearce, 1998; Singh & Creamer, 1991). There are three possible ways in which whey protein-casein interactions can occur, as suggested by Oldfield, Singh, and Taylor (1998) and Oldfield, Singh, Taylor, and Pearce (2000); (i) direct interaction between unfolded reactive monomeric β-lg and κ-casein, (ii) interaction of β-lg aggregates with κ-casein and (iii) interaction of α-La with κ-casein via β-lg- α-La aggregates. β-lg associate with casein micelles directly via binding with κ-casein, but α-La first form an intermediate

with β-lg in the solution before being associated with casein micelles (Corredig & Dalgleish, 1999). Oldfield, Singh, and Taylor (1998) and Vasbinder and De Kruif (2003) studied the association of whey proteins with casein micelles at pH 6.35 to 6.9 in heated skim milk. They found that above pH 6.6 there was partial coverage of casein micelles as well as whey protein aggregates were formed; below pH 6.6 all whey proteins in solution were associated with casein micelles. Whey proteins can also bind with dissociated κ-casein in the milk serum and in addition to that whey protein- κ-casein complexes can dissociate from casein micelle and release in the milk serum (Donato & Guyomarc'h, 2009).

Minerals play a significant role in the stability of milk protein system during heat treatment. The main milk salts are calcium phosphate and calcium citrate. Ca^{2+} in milk affect the denaturation temperature of β-lg and promote its aggregation by attaching to β-lg, this process is enhanced by the presence of Mg^{2+} (Xiong, 1992; Zhu & Damodaran, 1994). Grandison (1988) reported that even very small increase in the concentration of Ca^{2+} and Mg^{2+} in milk markedly reduces run time of UHT plant, although they attributed the sharp reduction in run time to addition of divalent cations immediately before UHT processing of milk, without enough time given to equilibrate throughout the different phases of the milk, this can disrupt delicate equilibrium of calcium phosphate and other minerals in serum phase and casein micelles.

Formation of insoluble calcium phosphate in milk upon heating lowers its pH as described by Visser and Jeurnink (1997).

$$Ca^{2+} + H_2PO_4^- \longrightarrow CaHPO_4\downarrow + H^+$$

This decrease in pH can promote casein-whey interactions as discussed earlier. The insoluble calcium phosphate formed during heating may associate with CCP of casein micelles or with β-lg aggreagtes in the serum phase or with both and finally get deposited on heat exchanging surfaces (Visser & Jeurnink, 1997). The precipitation of minerals is partly driven by the temperature difference between the process fluid and the heat exchanging surface (Jeurnink, Walstra, et al., 1996).

It is important to note that this reduction in pH does not increase the solubility of calcium phosphate due to the fact that calcium phosphate loses solubility at high temperature (Deeth & Lewis, 2017). The loss of calcium phosphate solubility dominates this phenomenon and reduces the soluble calcium in the milk leading to low Ca^{2+} levels along with low pH at UHT temperatures (On-Nom, Grandison, & Lewis, 2012).

2.7 Product and processing factors affecting heat stability of milk proteins and fouling of heat exchanging surfaces

The fouling of heat exchangers during UHT treatment of milk protein products can be influenced by properties of the product being processed and the processing conditions of the UHT plant.

2.7.1 Product factors

The first major factor in UHT fouling is the composition of the product, which cannot be easily changed in the case of milk (Bansal & Chen, 2006). In contrast, the composition of RTD high protein products can be formulated to achieve desired heat stability during UHT processing, but because milk proteins are major ingredients of these products, the problems associated with heat stability of milk proteins are still encountred. The difference in protein properties depending upon its source and batch to batch variability also plays a role in UHT stability of the product (Sikand et al., 2010). RTD high protein beverages may differ from milk in composition on the basis of the type and concentration of minerals, saccharides and fat content. Changes in the composition of milk protein product can change the composition of fouling deposits as demonstrated by Christian, Changani, and Fryer (2002). They studied the effect of the addition of calcium and phosphorous on fouling of WPC solutions used as a model fluid. Their study showed that UHT processing of modified WPC solutions resulted in the formation of deposits with higher mineral content, when the mineral content of model fluid was increased.

Most of the RTD high milk protein products are formulated from reconstituted milk powders. Reconstituted milk powder is less susceptible to fouling, mainly attributed to already denatured β-lg (almost 25%) during powder manufacture process (Changani et al., 1997; Visser, Jeurnink, Shraml, Fryer, & Delplace, 1997). Formulation of RTD high protein beverages may lead to a concentrated product with high total solid content. The concentration of milk leads to a dense packing of milk solids and increased viscosity of the product, which in turn decreases the heat stability of milk concentrates and make these products more susceptible to storage defects (Hinrichs, 2000). In addition to that, a concentrated product can be too viscous to pass through a UHT processing unit. Effect of concentration of total solids (TS) on the fouling behaviour of RSMP during UHT processing has been studied by Kastanas (1996) in the range of 9 to 30 % TS and by Prakash (2007) in the range of 10 to 20% TS. Both of these studies showed increased fouling with an increase in TS. Protein heat stability and solubility are highly affected by protein concentration. High protein content results in shorter distances between protein molecules, which in turn cause more protein-protein interactions and associations (Çakır-Fuller, 2015; Sağlam, Venema, De Vries, & Van Der Linden, 2014). When

high protein dispersions are heat treated the exposed hydrophobic and sulfhydryl groups result in protein aggregation.

Prakash (2007) showed that increase in whey protein content of 10% TS RSMP to match the whey protein content of 15 and 20% TS RSMP greatly reduced its UHT heat stability, While RSMP with 20% TS showed significant improvements in UHT heat stability, when total calcium of RSMP was reduced. Furthermore Fickak et al. (2011) demonstrated that an increase in protein content of whey protein solution caused an increase in the extent of its fouling. The rate of β-lg aggregation increases with more frequent interactions between whey protein particles. Studies on heat induced denaturation of β-lg showed that protein concentration influenced the aggregation. The average molecular mass of heat induced β-lg aggregates increased with increasing initial protein concentration (Hoffmann, Sala, Olieman, & de Kruif, 1997; Le Bon, Nicolai, & Durand, 1999). In contrast Crowley, Megemont, et al. (2014) studied heat stability of RMPCs (prepared from MPC powders with varying concentrations of protein content, MPC35 to MPC90) at 140 ^{0}C as a function of pH from 6.3 to 7.3. Their results showed that even the protein content of all the RMPCs was kept same, the relative abundance of other milk constituents i.e. lactose and minerals strongly affected the pH-HCT behaviour of the RMPC. RMPC35 was more heat stable at all the pH values than RMPC90, even though the TS content of RMPC35 was almost 2.5 times greater than the RMPC90. These studies demonstrate that compositional factors and nature of milk protein system of the product are more crucial than TS concentration for deposit formation during thermal processing of milk protein products.

The milk protein system is very sensitive to pH changes of the product (Crowley et al., 2015; Crowley, Megemont, et al., 2014). In general, the amount of fouling increases at acidic pH, when pH of solution approaches near isoelectric point (pI) of milk proteins, decreasing repulsions between protein molecules and causing proteins to coagulate (De Jong, 1997; Sauer & Moraru, 2012). In addition to that, concerning mineral salts, pH affects Ca^{2+} level in milk. When pH is reduced from 6.95 to 6.35, there is almost 2.5 times increase in Ca^{2+} levels in cow's and goat's milk (Zadow, Hardham, Kocak, & Mayes, 1983). The increase in Ca^{2+} may be the consequence of increased solubility of casein micelle bound calcium at low pH and its release in the serum phase (Huppertz, 2016). This can lead to the formation of insoluble calcium phosphate upon heating as discussed in the previous section. The calcium phosphate can precipitate in the bulk fluid and onto the milk proteins surfaces eventually forming deposits on the heat exchanging surfaces. The pH influences the the amount of fouling deposits and it is also responsible for the composition of deposits, shifting from mainly proteinaceous to minerals or vice versa (Visser et al., 1997).

Another important product factor that can be significant in fouling of milk protein dispersions is dissolved air in the bulk fluid. When the product is heated, the solubility of air decreases. Dissolved air can only encourage fouling, if it forms bubbles on the heating surface (De Jong, 1997). These air bubbles can act as nuclei at the heat transfer surface for formation of deposits. A tiny air bubble causes dry surface under it and there is a large temperature gradient between heat transfer surface and bulk fluid flowing over the air bubble, which results in evaporation of water near surface and its condensation near air/liquid interface at the location of air bubble (Jeurnink, Walstra, et al., 1996). If the air bubble collapses, the concentrated material at the bottom of the air bubble deposits on the heating surface, which contributes to fouling. Air bubbles can shift the composition of fouling deposits to higher casein content, due to tendency of caseins to adsorb at the air-liquid interface in foams (De Jong, 1997; Jeurnink, 1995; Jeurnink, Walstra, et al., 1996).

2.7.2 Processing factors

Temperature is undoubtedly the most important factor controlling fouling, either bulk fluid temperature, heating medium temperature, the temperature gradient between heating medium and bulk fluid (ΔT) or the time/temperature combinations used during preheating and sterilization of UHT product (Belmar-Beiny, Gotham, Paterson, Fryer, & Pritchard, 1993; Burton, 1968; Prakash, Kravchuk, & Deeth, 2015).

As already discussed increased temperature during UHT processing of milk causes conformational changes in protein structures and affect the mineral system of the milk. This leads to instability of milk constituents and deposit formation on heating surfaces. Nature of fouling deposit, Type A or Type B also depends upon the processing temperatures being used (Burton, 1968). Researchers have found that, there is a decrease in milk protein stability (Sauer & Moraru, 2012) and an increase in fouling (Kastanas, 1996) with increased processing temperatures. Very high temperatures of processing may cause a large temperature gradient in product and heating medium and fouling is increased with increased ΔT (Jeurnink, Walstra, et al., 1996).

There is a general observation of a decrease in fouling in sterilization section of UHT plant by increasing the severity of preheating of milk products (Burton, 1968; Lalande et al., 1984; Prakash et al., 2015). On the other hand, Srichantra, Newstead, McCarthy, and Paterson (2006) observed that higher preheat treatment resulted in higher fouling rates in fresh milk, reconstituted WMP (RWMP) and recombined milk. They linked homogenisation of their milk products prior to processing with increased fouling rates.

Prakash et al. (2015) did an elaborated study on the effect of preheat and UHT temperatures on deposit formation in a UHT plant. They found that during UHT processing of RSMP, longer preheating time (25 s) coupled with high preheating temperature (95 ^{0}C) caused a longer run time of an indirect UHT plant as compared to lower time/temperature combinations. This decrease in deposit formation may be because of almost complete aggregation of denatured β-lg in the bulk fluid in the preheating section, which reduces the deposition of sticky β-lg on the heated surfaces in the high heat section. The other explanation of reduced fouling may be the formation of small molecular weight soluble whey protein aggregates in the bulk fluid, which do not easily tend to precipitate and deposit on the heated surfaces as compared to larger insoluble aggregates (Ryan, Zhong, & Foegeding, 2013). Prakash et al. (2015) also observed that there was a general trend of increased fouling with increase in UHT temperature (135 to 150 ^{0}C) when product was held for less time (5 s) at all the preheating temperatures, but when product was held longer (25 s) at preheating, there was decrease in deposit formation with increased UHT temperature (135 to 145 ^{0}C). However, when UHT temperature was further increased to 150 ^{0}C, run time of UHT plant markedly decreased due to excessive deposit formation. They attributed this sudden change in fouling behaviour to increased coagulation of caseins at very high temperatures.

Formation of deposits is also affected by the fluid velocity and the Reynolds number (Re) in a heat exchanger (Visser et al., 1997). Increasing the fluid velocity in heat exchanger reduces deposit formation; however flow rate is not a major factor in fouling as compared to other factors (Belmar-Beiny et al., 1993; Burton, 1994; Gordon, Hankinson, & Carver, 1968). Burton (1994) proposed that at low flow rates the thickness of the laminar sub-layer adjacent to the heating surface is greater, so that the volume of material subjected to a higher temperature for longer period of times is greater. By contrast at high velocities, there is a possibility of removal of material by shear forces, which may contribute to less fouling at a higher velocity, however mixing of these detached fouling particles with the product can affect the quality of the product and can also contribute to sedimentation during storage. Gordon et al. (1968) showed that there was decrease in fouling with increased flow rates in a tubular heat exchanger. In the case of plate heat exchangers (PHE), the flow rate cannot be easily related to fouling, because of the complex flow geometries involved (Changani et al., 1997).

Re depends upon density, viscosity and velocity of fluid and flow regimen of the heat exchanger (Bansal & Chen, 2005; Lalande & Tissier, 1985). An increase in concentration and viscosity of product can shift the flow from turbulent to laminar; while an increase in fluid velocity can change the flow conditions from laminar to turbulent. Belmar-Beiny et al. (1993); Guérin, Ronse, Bouvier, Debreyne, and Delaplace (2007) observed a decrease in fouling with increased turbulence in the bulk

fluid. Georgiadis and Macchietto (2000) modelled and simulated fouling of milk in PHE and they found that the temperature drop due to fouling is less at Re = 4500 (turbulent flow conditions) as compared to Re = 2700 (laminar-turbulent transition flow conditions).

The other important factor affecting fouling is the nature of the heat transfer surface, since fouling deposits build up on the walls of heat exchangers (Burton, 1994). However, when the heat transfer surface fouls charcteristics of the surface no longer affect fouling behaviour. Making heating surfaces resistant to fouling can be an easy way to minimize fouling. Standard heat treatment equipments in the food industry are made of stainless steel due to its resistance to corrosion and durability (Sandu & Lund, 1985). Surface energy, surface microstructure (roughness and other irregularities), presence of active sites, residual material from previous runs, types of stainless steel and presence of chromium oxide or passive layer, these all factors influences the fouling of stainless steel surfaces (Bansal & Chen, 2006; Beuf et al., 2003). A clean metal surface posses large free surface energy gradient. Protein are very surafce active and they will start to adsorb on the metal surface to reduce this free energy (Belmar-Beiny & Fryer, 1993; Santos et al., 2003). A rough surface can promote fouling by providing more surface to which material can adsorb and by allowing better attachment of the deposit layer to the surface (Burton, 1994).

Antifouling coatings of surfaces to reduce depost formation have been widely studied as reviewd by Bansal and Chen (2006); Mérian and Goddard (2012) and Sadeghinezhad et al. (2013). Ni-P-PTFE surface has been shown to accumulate least deposit buildup and easiest to clean among other antfouling coatings. It is regarded as the most promising surface coating to reduce nonmicrobiological deposits such as calcium phosphate and whey proteins (Sadeghinezhad et al., 2013).

2.8 Fouling reduction and improving heat stability of high milk protein beverages during UHT processing

The milk protein system is heat labile and its denaturation and aggregation during heat treatments cannot be completely prevented. This thesis focuses on possible interventions that can be used during the formulation of high protein dispersions in order to improve their UHT stability and reduce fouling of heat exchanging surfaces. Improved UHT stability of these products can be achieved by choosing a milk protein ingredient or milk protein powder mix based on knowledge about milk protein heat stability as discussed in previous sections. Differences in protein ingredient properties depending upon their production method, compostion, protein profile and batch to batch variability play a role in UHT stability of the product (Sikand et al., 2010). Formulators can attempt to blend different milk powders such as casein and whey protein ingredients to achieve a modified protein and mineral profile for maximum heat stability of the processed product.

Many researches have documented the protective effect of caseins to prevent thermal aggregation of whey proteins (Bhattacharyya & Das, 1999; Guyomarc'h, Nono, Nicolai, & Durand, 2009; Liyanaarachchi, Ramchandran, & Vasiljevic, 2015; Morgan, Treweek, Lindner, Price, & Carver, 2005; Parkinson & Dickinson, 2004; Yong & Foegeding, 2008). They attributed this behaviour of caseins to their molecular chaperone like activity to protect whey proteins from heat induced stresses. Molecular chaperons have the ability to stabilise proteins against denaturation, aggregation under various stresses such as high temperatures (Morgan et al., 2005). The chaperone behaviour of caseins has been extensively reviewed by Yong and Foegeding (2009).

Most of the studies related to chaperon activity of caseins are performed on individual casein molecules and utilization of individual caseins for their chaperon activity is impractical on an industrial scale; use of milk protein ingredients such as MPC, MCC and caseinate would be more suitable. Guyomarc'h et al. (2009) studied the heat induced aggregation of whey proteins from WPI in the presence of κ-casein or sodium caseinate, they found that presence of sodium caseinate and κ-casein resulted in smaller whey proteins aggregates, when heated at neutral pH. However, the protective effect of sodium caseinate was not as much pronounced as κ-casein, they attributed this behaviour to competition between α-caseins, β-casein and κ-casein of sodium caseinate for interaction with β-Lg. Recently Liyanaarachchi et al. (2015) have studied the effects of inclusion of caseins on properties of heat induced aggregates of whey proteins. They used MPC and WPI to achieve different casein-whey protein ratios. They concluded that increased pH and casein content in casein-whey protein formulations helped to form smaller nano-sized whey protein particles, whereas reduced casein content resulted in micrometre-sized larger whey protein aggregates. However, there was no positive effect of casein inclusion on the reduced particle size of whey protein aggregates, when heated at a pH near pI of whey proteins. These findings provide possibilities of formulating food products using caseins and whey proteins in suitable ratios, where caseins can help to prevent thermal aggregation and formation smaller nano-sized soluble aggregates of whey proteins (Liyanaarachchi et al., 2015; Yong & Foegeding, 2009). This will result in a beverage that can have a longer shelf life without excessive sedimentation. Moreover, caseins themselves are very stable at high temperatures (Dalgleish & Corredig, 2012; Douglas Jr, Greenberg, Farrell Jr, & Edmondson, 1981).

Milk minerals greatly influence the heat stability of milk (Horne & Muir, 1990). However the mineral balance of MPC and WPC powders is altered during their manufacturing process (De la Fuente, 1998). The modified mineral profile of protein dispersions prepared from these protein ingredients can negatively or positively affect their UHT stability (Crowley et al., 2015). Therefore the selection of milk protein powders based on their mineral profile can be done to achieve a UHT stable product.

The other approach to modify the mineral balance of milk protein dispersion is to add some mineral salts. Sulfhydryl reactive substances such as potassium iodate can be added to a product to improve its heat stability, which reduces the formation of high molecular weight aggregates during UHT processing due to the ability of potassium iodate to oxidise the heat activated sulfhydryl groups in denatured proteins(Skudder et al., 1981). Addition of calcium chelating agents such as phosphates and citrates can also greatly improve the heat stability of milk proteins by modifying pH and Ca^{2+} of the milk products(Deeth & Lewis, 2015). The use of phosphates in UHT milk is permitted by some countries, including the European Union (Deeth & Lewis, 2017).

2.9 Conclusions

High protein beverages possess excellent physiological properties and have significant commercial value. This literature review summarises the importance of studying protein stability in UHT processed high protein beverages in order to control and stabilise milk proteins against UHT processing. Milk protein ingredients are used in the formulation of high protein beverages, however milk protein system is not very stable at UHT temperatures. There is a problem of casein coagulation, whey protein denaturation and aggregation, and milk mineral precipitation during UHT processing. This can cause fouling of heat processing surfaces, which is a serious problem in the dairy industry. There is sufficient literature that deals with UHT fouling of milk, however there is a lack of research in UHT stability of reconstituted milk protein ingredients at high protein concentration. Therefore, these is a need for research that can study UHT stability of milk protein dispersions at high protein content similar to beverage mixes used in commercial products. The UHT processing of these products can be improved by studying the fouling behaviour of reconstituted milk protein ingredients such as MPC and WPC. The beverage formulations can be modified to achieve a protein profile that can positively affect their UHT stability. Controlling denaturation and aggregation of whey proteins using chaperone activity of caseins is a promising technique. The study of interactions of milk proteins with themselves and other ingredients such as flavouring agents, milk minerals, stabilisers etc. during UHT processing is of high commercial value in order to minimize production costs.

CHAPTER 3: COMPARISON OF ULTRA-HIGH TEMPERATURE (UHT) STABILITY OF HIGH PROTEIN MILK DISPERSIONS PREPARED FROM MILK PROTEIN CONCENTRATE (MPC) AND CONVENTIONAL LOW HEAT SKIMMED MILK POWDER (SMP)

The following publication has been incorporated as Chapter 3:

Singh, J., Prakash, S., Bhandari, B., & Bansal, N. (2019). Comparison of ultra-high temperature (UHT) stability of high protein milk dispersions prepared from milk protein concentrate (MPC) and conventional low heat skimmed milk powder (SMP). Journal of Food Engineering, 246, 86-94.

Contributor	**Statement of contribution**
Jaspal Singh (Candidate)	Concept and design (85%) Analysis and interpretation (70%) Drafting and production (55%)
Sangeeta Prakash	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (10%)
Bhesh Bhandari	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (10%)
Nidhi Bansal	Concept and design (5%) Analysis and interpretation (20%) Drafting and production (25%)

Abstract: This study compared the UHT (145 ºC for 5 s) stability and fouling behaviour of high protein milk dispersions prepared from reconstituted low heat skimmed milk powder (RSMP) and milk protein concentrate powder (RMPC). It was found that RMPC at 10 and 14% protein content was more UHT stable as compared to lower protein content RSMP (3.25, 6.5, 7, 7.5 and 8%). Matching the total solids and mineral composition of 7.5-RMPC with 7.5-RSMP by addition of minerals and lactose markedly reduced its UHT stability (UHT run-time reduced to 66 min from >120 min). The RP-HPLC analysis showed increased casein dissociation but similar whey protein aggregation in 7.5-RSMP as compared to 14-RMPC. UHT processing lead to formation of larger particles in case of 7.5-RSMP (1.84 μm D(0.9)) as compared to 14-RMPC (0.23 μm D(0.9)). It was observed that the mineral environment affected protein interactions leading to the differences in UHT behaviour of RSMP and RMPC.

3.1 Introduction

Increased consumer awareness towards the benefits of protein in the diet and its positive effects on weight loss and muscle building has surged the demand for high protein weight loss diets (Friedman, 2004). There are various formats of high protein processed foods available in the market, but ready-to-drink (RTD) high protein beverages are a notable departure from typical powder type sports supplements specifically targeted at body builders and sports people (Baxter et al., 2011). Due to their convenience, high protein beverages are also appealing to health conscious ordinary consumers. RTD high protein beverages based on milk proteins are processed liquid food products. Manufactures combine high protein dairy powders along with other ingredients to obtain a ready to drink product with desired protein, fat and carbohydrate content, amino acid profile and sensory attributes (Baxter et al., 2011; Jelen, 2011). The RTD high protein beverages are required to contain high protein levels without compromising product stability and quality. Food and drug administration (FDA) of the United States requires adding minimum 10 g protein per 240 mL of drink (~ 4.2%) to claim high protein beverage (Etzel, 2004).

There are several types of milk protein ingredients available in the market. Skim milk powder (SMP), whole milk powder, milk protein concentrate powders (MPC), casein and whey protein concentrate powders are widely produced and used as ingredients in a range of milk protein based beverages. MPCs are complete dairy proteins, containing casein and whey proteins in their original proportions found in milk and in their native state. Much of the caseins in concentrates are in micellar form and whey proteins are largely undenatured (Agarwal et al., 2015). The protein content of MPC varies from 42 to 85%, which is indicated by the number following MPC, e.g. MPC85. MPCs find application in the manufacture of food emulsions, cheese, yogurt, ice cream, health related products and various other dairy products (Kelly, 2011). There is growing popularity of MPC as a protein source in neutral pH RTD high protein beverages due to the fact that it is an excellent source of protein and can provide a milky flavour and opacity to the drink (Agarwal et al., 2015). MPC are manufactured from skim milk by ultrafiltration and diafiltration process, followed by spray drying the retentate (Havea, 2006). The filtration process also partially removes lactose and mineral salts from the milk. Due to the significant differences in the composition of non-protein constituents of MPC and SMP, reconstituted MPC (RMPC) can provide more protein per total solids as compared to reconstituted SMP (RSMP) (Deeth & Hartanto, 2009). However, RMPC contains altered mineral environment as compared to RSMP due to ultrafilteration and diafiltration process used to concentrate milk proteins during MPC manufacturing process. MPC contains more calcium than SMP, however per unit of protein MPC contains less calcium (Kelly, 2011).

Generally, There are two types of high protein beverages based on milk protein ingredients: neutral pH (pH~6.8) and low pH acidic beverages (Beecher, Drake, Luck, & Foegeding, 2008a). MPC based beverages are mostly neutral pH beverages due to its high casein content (Agarwal et al., 2015). Neutral pH beverages are required to be commercially sterilized to make them shelf-stable for a longer storage period. UHT is a commonly used technology for thermally processing these products. There are less colour and flavour changes and minimal losses of nutrients during UHT due to very short holding time and faster heat transfer as compared to retort sterilisation (Burton, 1994). However, thermal processing of dairy products causes the formation of deposit layers on heat transfer surfaces, which is known as fouling (Sadeghinezhad et al., 2013). Fouling is a result of heat-induced destabilisation of milk constituents during processing which can limit the processing time and incur costs of cleaning and processing down times. Fouling may also adversely affect product quality due to dislodgement of deposits and mixing with product. Fouling deposits has very low heat conductivity as compared to process surfaces and fouling layers can reduce the heat flow, which causes insufficient processing of product (Prakash et al., 2005). In addition, increased obstruction to fluid flow can cause pressure drops across the processing line. These issues may increase the energy requirements due to increased energy costs to maintain adequate processing condition. In the worst scenario UHT processing plant may also be required to be shut down for cleaning (Bansal & Chen, 2006). There are several factors affecting fouling of heat transfer surfaces which can be broadly classified as product and processing factors (Deeth, 2010). Understanding the behaviour of a milk product during UHT processing can be of great importance in controlling fouling and increasing run-time of the processing plant. Milk protein system consists of different types of proteins and a complex mixture of native whey proteins, whey protein aggregates and whey protein-casein complexes can be formed during heating of milk protein dispersions (Wijayanti et al., 2014).

The heat stability of milk proteins and their susceptibility to denaturation and aggregation and final composition of this mixture depends on the temperature and time of heating, pH, the relative abundance of other proteins and salts in the food system (Singh, 2004). The difference in thermal stability of proteins coming from different milk protein ingredients can play an important role in determining the UHT stability of the final product (Sikand et al., 2010). In the case of high protein beverages, improved UHT stability can be achieved by choosing a milk protein powder based on knowledge about their heat stability. The differences in the composition of SMP and MPC can cause these two protein dispersions to behave differently during UHT processing. A lot of research has been conducted on UHT stability of normal strength and concentrated RSMP, but not sufficient work has been previously reported on UHT processability of RMPC. More research is required on UHT

stability of RMPC because of its increasing usage in the formulation of UHT processed RTD high protein beverages (Agarwal et al., 2015).

The present work is focused on understanding the behaviour of high protein milk dispersions prepared from MPC and compare with conventional low heat SMP during UHT processing. An understanding of UHT stability and fouling behaviour of RMPC can be beneficial for controlling and minimizing heat induced fouling during the use of MPC in commercially sterilized high milk protein beverages, and other UHT treated products.

3.2 Materials and Methods

3.2.1 Materials

Commercial MPC and low heat SMP (purchased from Real Dairy Australia Pty. Ltd, Australia) were used in the preparation of reconstituted milk protein dispersions for all but one experiment for which MPC-G from a different manufacturer was purchased from Maxum Foods, Queensland, Australia for comparison purposes. Lactose was procured from Bio-Strategy Laboratory Products Pty. Ltd., Queensland, Australia. Standards for pure proteins were bought from Bio-Rad, Australia. Other chemicals and reagents were purchased from Sigma-Aldrich Pty. Ltd., NSW, Australia unless otherwise stated. Simulated milk ultrafiltrate (SMUF) was prepared according to the recipe by Jenness (1962).

3.2.2 Compositional and quality analysis of milk powders

MPC and SMP were analysed for total protein content and lactose content using Kjeldahl method (AOAC, 2005) and titrimetric method (AS, 1994), respectively. MPC and SMP were also analysed for mineral composition using Inductively Coupled Plasma-Optical Emission Spectrometric (ICP-OES) analysis as described by Martinie and Schilt (1976).

MPC was also analysed for its solubility according to Bansal, Truong, and Bhandari (2017). MPC solubility was analysed at rehydration temperature of 50 °C, which was the temperature used for reconstitution of samples throughout this study. The solubility of each sample was calculated as follows:

Solubility (%) = (total solids per g of filtrate/ total solids per g of suspension) * 100

3.2.3 Undenatured whey proteins and electrophoresis of milk powders

Undenatured whey proteins were quantified by adjusting the pH of RMPC and RSMP to 4.6 using 2M HCl or 2M NaOH, followed by centrifugation at 4500 *g* at 20 ºC for 15 min to precipitate serum

caseins and denatured soluble whey proteins (García-Risco, Ramos, & López-Fandiño, 1999). Supernatants were analysed for protein content using Kjeldahl method (AOAC, 2005). All measurements were performed in duplicates. Undenatured whey protein content was reported as a percentage of the total protein content of RMPC and RSMP.

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis was performed under reducing (R SDS-PAGE) and non-reducing (NR SDS-PAGE) conditions following the method of Laemmli (1970). Precast polyacrylamide gels (4-20%), sample buffer and Precision Plus Protein™ Dual Xtra molecular weight standard were obtained from Bio-Rad Laboratories Pty. Ltd, NSW, Australia. All other preparations for SDS-PAGE analysis were based on the standard guidelines in the Bio-Rad manual (Catalog number 161-0993. Samples were mixed 1:1 with 2X sample buffer for NR SDS-PAGE analysis. For R SDS-PAGE analysis, samples were mixed with sample buffer containing 10% β-mercaptoethanol and heated at 95 ºC for 5 min. 10 µg protein was loaded onto each well. Electrophoresis was carried out at 80 V for 30 min and then at 100 V. Bio-Rad Mini Protean Tetra Cell system (Bio-Rad Laboratories Pty. Ltd, NSW, Australia) was used to run the gels. The gels were stained overnight with a solution of 0.04% Coomassie Brilliant blue G250, 25% methanol and 10% acetic acid in water. The gels were scanned and analyzed using Bio-Rad GS-800 Calibrated Densitometer (Bio-Rad Laboratories Pty. Ltd, NSW, Australia).

3.2.4 Preparation of reconstituted milk protein dispersions

Calculated amounts of milk powders, lactose, SMUF and distilled water were mixed to achieve required (w/w) protein content and total solids of RMPC and RSMP. Table 3.1 shows different samples and sample codes used in this study. Suffix Unheated and UHT were used to denote unheated and UHT heated samples, respectively. Reconstituted protein dispersions were prepared by reconstituting milk powders in distilled water at 50±2 ºC. The protein dispersions were kept under refrigeration overnight (~14 h) to ensure complete hydration of all powder particles. Protein dispersions were then allowed to reach room temperature. The pH of protein dispersions were analysed and adjusted to 6.8 using 2M NaOH or 2M HCl, if required. Milk protein dispersions were filtered to remove any undissolved particles.

Table 3.1: Description of reconstituted milk protein dispersions used.

Sample	Ingredients	Protein content (%w/w)	Total solids (% w/w)
3.25-RSMP	SMP	3.25	10.00
6.5-RSMP	SMP	6.50	20.00
7-RSMP	SMP	7.00	21.53
7.5-RSMP	SMP	7.50	23.07
8-RSMP	SMP	8.00	24.61
10-RMPC	MPC	10.00	12.27
14-RMPC	MPC	14.00	17.17
16-RMPC	MPC	16.00	19.63
14-RMPC-G	MPC-G	14.00	17.17
7.5-RMPC-LAC	MPC and Lactose	7.50	23.07
7.5-RMPC-LAC-SMUF	MPC, lactose and mineral salts	7.50	23.07

3.2.5 Ethanol stability of reconstituted milk protein dispersions

Ethanol stability was determined by mixing equal volume (2 mL) of milk and a range of ethanol solutions (50 to 100% at 2% intervals) and carefully examining the sample for clotting when poured in a petri dish. The highest concentration of ethanol, which did not cause coagulation, was reported as ethanol stability for the sample.

3.2.6 Ionic Ca activity in reconstituted milk protein dispersions

Ca-ion activity in milk protein dispersions was measured using LAQUAtwin calcium ion meter (Horiba Instruments, Japan). The calcium ion meter was calibrated using 3.74 mM (150 ppm) and 49.90 mM (2000 ppm) Ca-ion activity standard solution before each experiment, according to manufacturer instructions. All measurements were performed at room temperature (22-23 ºC).

3.2.7 UHT processing of reconstituted milk protein dispersions

The samples were UHT processed using a bench top UHT plant as shown in Fig 3.1. The product temperature at inlet and outlet of sterilization section was measured by T-type thermocouples, which were connected to a data logger and the temperature data was recorded in a Microsoft Windows based data acquisition system, VISIDAQ (PCLD-8115, Advantech Co., Ltd., Taiwan). A complete description of bench top UHT plant can be found in Prakash (2007). The internal diameter of stainless

steel tube carrying the sample through UHT heating and holding sections was 0.003 and 0.0046 m respectively. The length of this tube at UHT heating and holding sections was 12 and 0.8 m respectively. The fluid flow through the UHT plant was turbulent (Re > 10500) for all the UHT runs performed for this thesis (Hasting, Blackburn, & Crowther, 2001; Pearce et al., 2001).

The volumetric flow rate of the product in the beginning of the trials for this chapter was 150 mL/min (2.5×10^{-6} m^3/s). The product was preheated to 95 °C and held at this temperature for 8 s in the holding tube before heating to 145 °C in sterilization section and held at this temperature for 5 s. Indicators used to end the UHT run due to deposit formation were as described by Prakash (2007). The UHT run was stopped if the back pressure could not be maintained at 0.4 MPa and high back pressure triggered the over pressure valve. The experiment was also stopped in case the outlet temperature of the sterilization section dropped below 120 ºC. The other unlikely scenario to stop UHT run was blockage of product channel due to severe fouling. Unless otherwise stated, if none of the above factors stopped UHT processing, the experiment was terminated after 120 min has elapsed into the UHT run. All experiments were performed in duplicate and their average value is reported.

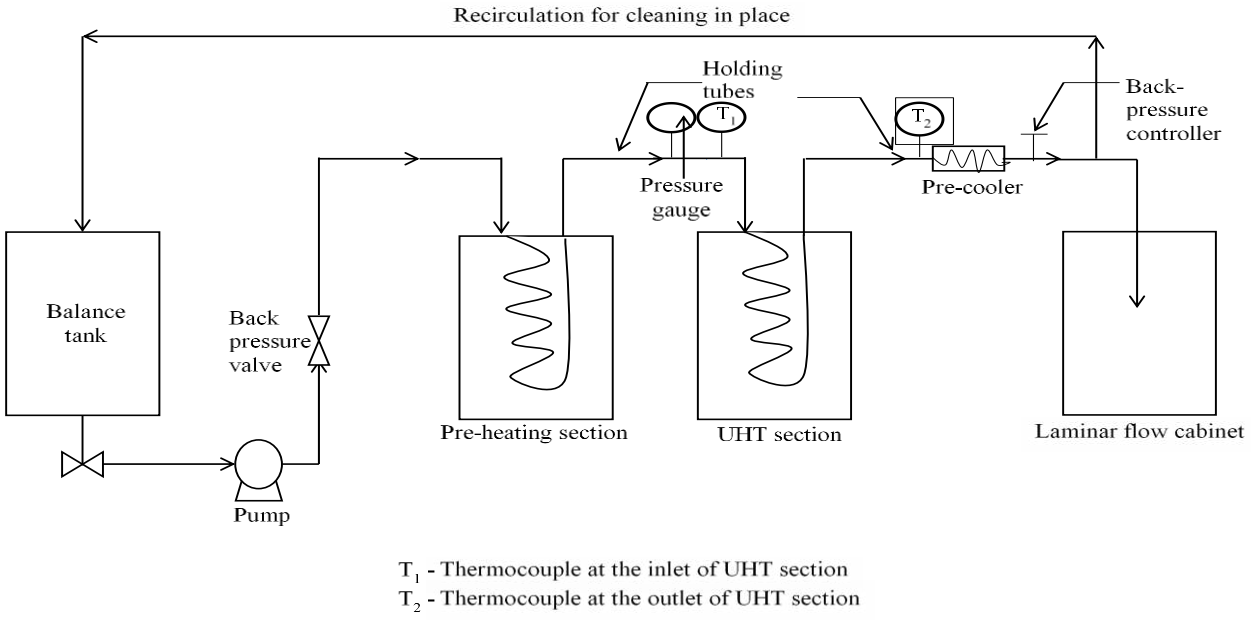

Figure 3.1: Flow diagram of bench top UHT plant.

3.2.8 Fouling measurements

Changes in overall heat transfer coefficient (OHTC) were used to monitor fouling. The plot of OHTC versus run time of UHT plant from the start to the end run was used to monitor the development of fouling during the UHT run. Equation 3.1 was used to calculate OHTC.

$$OHTC = \frac{GC_p\Delta\theta}{A\Delta T_{lm}} \qquad \text{eq (3.1)}$$

Where, G is the mass flow rate of the product in kg/s; C_p is the specific heat of product in J/kg^{0}C; $\Delta\theta$ is the temperature difference between the inlet and outlet of the UHT section, in ^{0}C; A is the heat exchanging surface area of the tubing in m^2; ΔT_{lm} is the log mean temperature difference (LMTD) in ^{0}C calculated using the equation 3.2.

$$\Delta Tlm = \frac{(T_o - T_{mo}) - (T_o - T_{mi})}{\ln[(T_o - T_{mo})/(T_o - T_{mi})]} \qquad \text{eq (3.2)}$$

Where T_o is the temperature of oil bath in ^{0}C; T_{mi} and T_{mo} are temperatures of milk at the inlet and outlet of the sterilization section in ^{0}C.

Specific heat and density of reconstituted milk powders were calculated using the specific heat and density of protein, carbohydrate, fat, ash and water and mass fraction of these major components in the dispersion (Choi, 1986; Singh, 2006).

3.2.9 Heat coagulation time measurements

Heat coagulation time (HCT) of samples was measured using the method described by Davies and White (1966) with some modifications. The measurement temperature was similar to UHT sterilization (145 ^{0}C). Glass vials (22.6 x 75.5 mm) containing 2 mL of sample were placed on a rocker and immersed in a temperature controlled oil bath for heating. The rocker speed was kept at ~8 revolutions per min. HCT was reported as time elapsed between putting the samples in the oil bath and appearance of first visible signs of coagulation.

3.2.10 Particle size distribution

Particle size distribution (PSD) of unheated and UHT processed protein dispersions were measured by dynamic light scattering (DLS) using a Malvern Mastersizer 2000MU-A (Malvern Instruments Ltd, Malvern, United Kingdom) as described by Dumpler and Kulozik (2016). The refractive index of protein was set at 1.41 and for dispersant (distilled water) was 1.33. Particle absorption index was kept as 0.001. Stirrer speed was set at 2000 rpm and laser obscuration was maintained between 10 and 11 during measurement. All measurements were performed at room temperature (23 ^{0}C).

3.2.11 Viscosity measurements

The apparent viscosity of unheated and UHT processed protein dispersions was measured using an AR-G2 Rheometer (TA Instruments Ltd., USA) equipped with 60 mm parallel plate geometry with

an interplate gap set at 300 μm during measurements. The temperature of the Peltier plate was set at 20ºC and viscosity measurements were performed after samples were allowed temperature equilibration for 1 min. Apparent viscosity at a shear rate of 300 s^{-1} was analysed because it normally falls under the typical range of shear rate encountered during pipe flow, mixing and stirring of liquid food products (Steffe, 1996).

3.2.12 Whey protein denaturation and heat induced dissociation of caseins

Unheated and UHT processed samples were ultra-centrifuged (Avanti JXN-30, Beckman Coulter, Australia Pty. Ltd., NWS, Australia) at 100,000g for 1 h at 20 ºC. The supernatant was removed carefully and analysed for non-sedimentable protein (NSP) content using the Kjeldahl method (AOAC, 2005). The supernatants were further analysed to quantify individual non-sedimentable proteins of interest using Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) (RP-HPLC-UV, Agilent 1100, Agilent Technologies Australia, Victoria, Australia) using method adopted from Wijayanti, Oh, Sharma, and Deeth (2013).

3.2.13 Statistical analysis

The data was analysed using Microsoft Excel and Minitab 16 software package. Significant differences between average values of replicate measurements on each data point was analysed by analysis of variance (ANOVA) using Tukey's HSD post hoc test at 95% confidence level.

3.3 Results and discussion

3.3.1 Compositional and quality analysis of milk powders

MPC contained on average 81.95 ± 1.77% (w/w) total protein and 6.26 ± 0.13% (w/w) lactose. The total protein and lactose contents of SMP on average were 32.64 ± 0.53% and 55.37 ± 0.53%, respectively. Amount of undenatured whey proteins in MPC (18.85 ± 0.55% w/w) and SMP (22.52 ± 0.21% w/w) were similar. This was also confirmed with SDS-PAGE analysis. Which showed that β-lactoglobulin (β-lg) and α-lactalbumin (α-la) were present in their native form in the milk powders as shown in Fig 3.2. Total calcium content of MPC (2.19 ± 0.05% w/w) was significantly higher than that of SMP (1.46 ± 0.05% w/w), presumably due to the higher protein (casein) content of MPC binding higher amount of colloidal calcium. The solubility of MPC was found to be 85.19 ± 1.61%, which reached 100% at 50 ºC. This ensured that the temperature used for reconstitution during this study was sufficient for complete rehydration of samples.

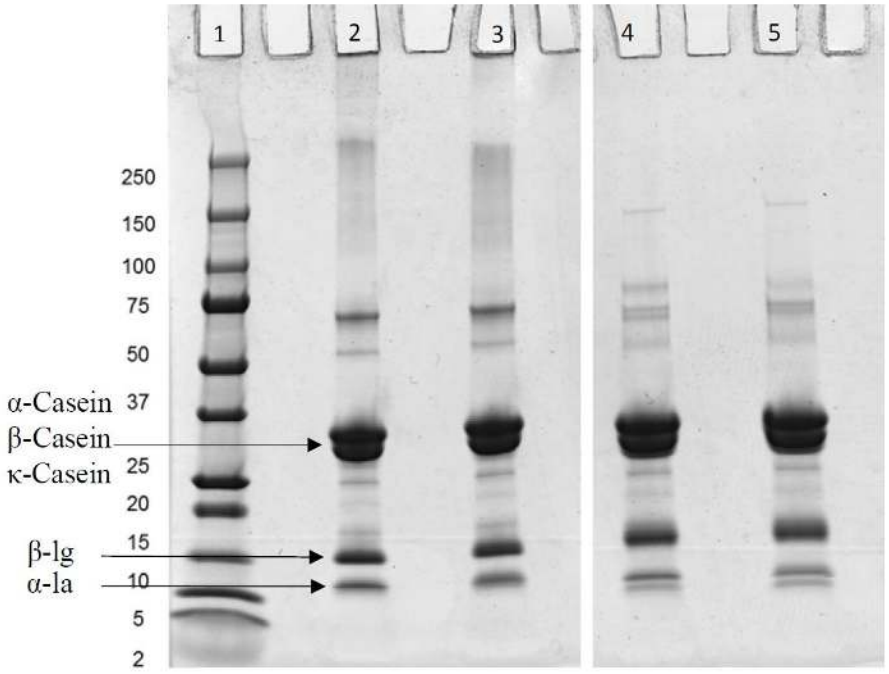

Figure 3.2: SDS-PAGE analysis of milk powders, Lane: 1. Molecular weight standards, 2. Low Heat SMP (Non-Reduced SDS-PAGE), 3. MPC (Non-Reduced SDS-PAGE), 4. Low Heat SMP (Reduced SDS-PAGE) and 5. MPC (Reduced SDS-PAGE).

3.3.2 Effect of protein concentration and type of milk protein ingredient on UHT Stability of milk protein dispersions

Firstly, a UHT processing trial was conducted with 3.25-RSMP to establish baseline performance of the bench-top UHT plant. The run-time for this sample was very long and UHT run was terminated after 300 min elapsed and ~50 kg sample was exhausted. The samples did not show any excessive pressure development and UHT temperature was maintained at 145±2 ºC. After that UHT stability of concentrated milk protein dispersions (RMPC and RSMP at different protein concentrations) was analysed. 10-RMPC and 14-RMPC were also very stable during UHT processing and average run-times of bench-top UHT plant exceeded 300 min for 10-RMPC and averaged 280 min for 14-RMPC. These samples were processed without any major temperature drops or pressure fluctuations. The RMPC sample with 16% PC was not UHT processed due to high viscosity and gel like consistency before processing. For further experiments, to be able to process multiple samples in a day, the UHT run was terminated after 120 min has elapsed, if fouling did not interrupt processing. Fig 3.3 and 3.4 shows average run times and changes in OHTC during UHT run, respectively, for all the milk protein dispersions used in this study.

For RSMP samples containing 6.5, 7 and 7.5% proteins, the UHT run lasted for 88, 72 and 23 min, respectively before fouling was observed. Excessive back pressure developed after this time, which

triggered the over pressure valve and the milk was pumped back into the balance tank of the UHT plant. Sample containing 8% proteins could not be processed through the UHT plant, because excessive back pressure developed as soon as 8-RSMP passed through the UHT section of the plant. This suggested that 8-RSMP was highly unstable under UHT conditions and fouled immediately. These results showed that total protein content in RSMP influenced its ability to be UHT processed. The UHT run decreased with increasing protein content. Although other studies do not report the effect of increased total protein directly, similar results were obtained by Kastanas (1996); Prakash (2007), when the total solids of milk were increased.

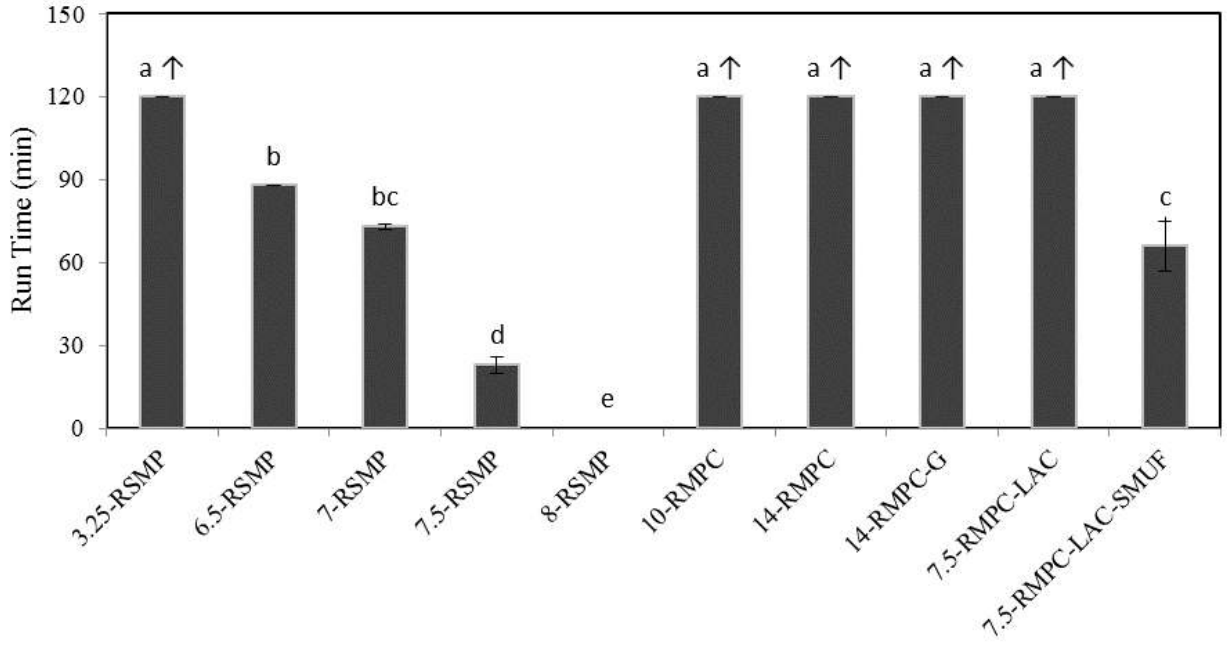

Figure 3.3: The average UHT run times on a bench top UHT tubular heat exchanger during processing of milk protein dispersions. Error bars represent standard deviation, n=2. Means with different letters are significantly different (P<0.05). Samples with ↑ did not foul in 120 min.

The RSMP containing 3.25% protein showed high OHTC values as compared to 6.5, 7 and 7.5-RSMP during UHT processing (Fig 3.4A). This can be attributed to low TS and high amount of water in the sample, which in turn leads to high values of specific heat (C_p) (Choi, 1986; Singh, 2006; Toledo, 2007). OHTC values are directly proportional to C_p as shown in eq. 3.1. On average, values of OHTC during the processing of 6.5-RSMP, 7-RSMP and 7.5-RSMP were lower than 3.25-RSMP. This also suggests that increased total solids and increased viscosity play a role in decreasing turbulence and heat transfer during UHT processing.

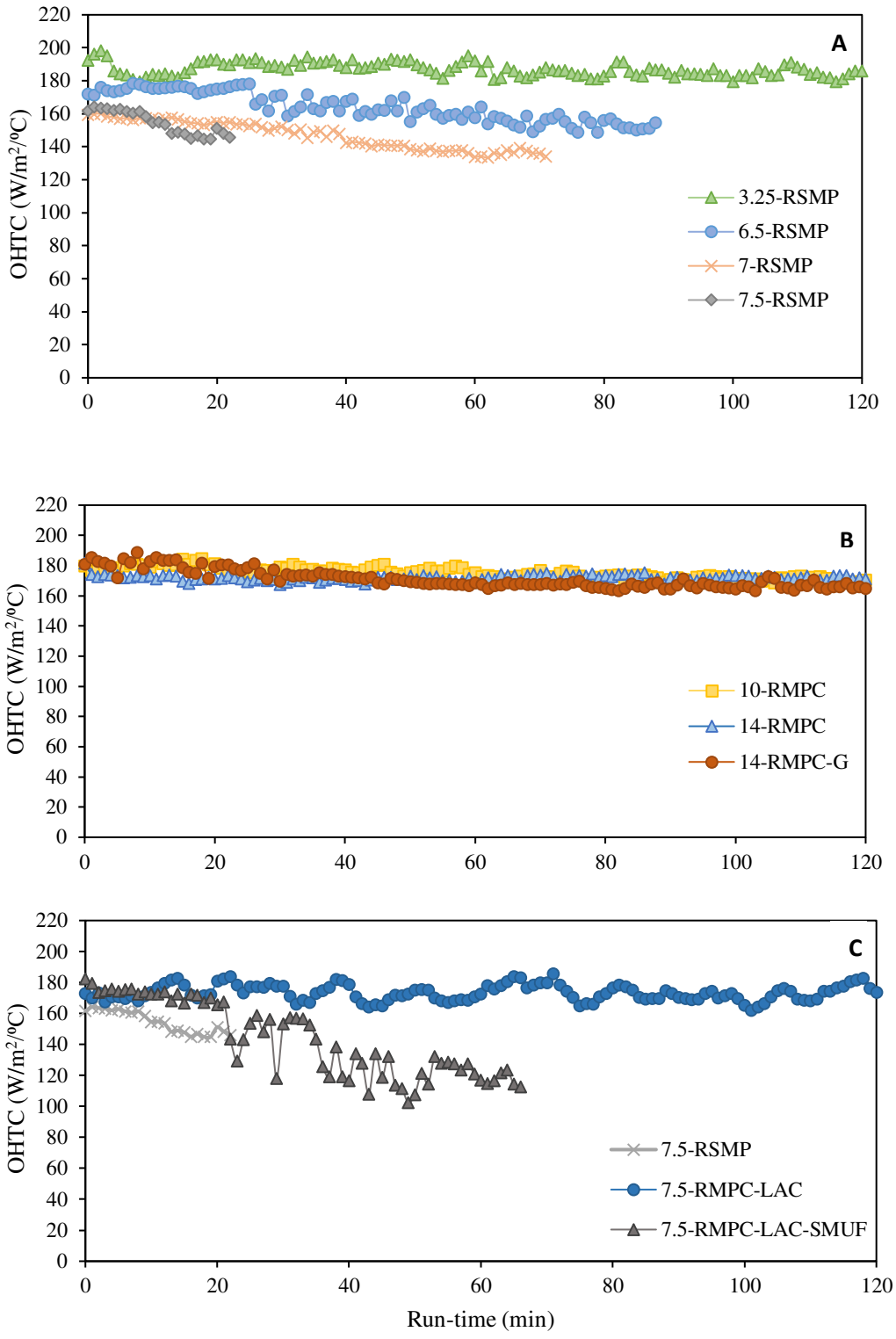

Figure 3.4: Variation in OHTC with run time for milk protein dispersions, (A) milk protein dispersions prepared using RSMP, (B) milk protein dispersions prepared using RMPC, (C) comparison of 7.5-RSMP with 7.5-RMPC with added lactose and SMUF. Average data of duplicate runs is presented here.

The OHTC vs run-time graph (Fig 3.4A) shows that the OHTC remained almost constant for 3.25-RSMP throughout the run, whereas, for concentrated RSMP samples there was a gradual decrease in OHTC with increasing run-time after an induction period. The observations for 3.25-RSMP were consistent with results previously reported by Prakash (2007). This OHTC vs run-time behaviour of concentrated RSMPs was due to gradual fouling of heat transfer surfaces with milk solids, which offered more resistance to heat transfer as compared to clean surfaces, causing lower UHT temperatures. A fouling induction period of 25, 14 and 8 min was observed for 6.5-RSMP, 7-RSMP and 7.5-RSMP, respectively suggesting that fouling started faster in samples containing higher protein and higher total solids

In concentrated RSMPs milk proteins are relatively densely packed as compared to 3.25-RSMP. Increased protein content in concentrated RSMPs increases the chances of interactions between protein molecules leading to an increase in the amount of higher molecular mass β-lg aggregates (Le Bon et al., 1999). This can result in an increased amount of voluminous Type A deposits (Tissier, 1984). These deposits will cause temperature drops and excessive fluctuations in back pressure to a point where back pressure could not be maintained at 0.4 kPa and UHT run had to be terminated. As 8-RSMP was extremely unstable to UHT processing, the effect of protein content higher than 8% could not be studied using RSMP.

Further, RMPCs were processed to observe the effect of increased protein content on UHT behaviour of high protein milk dispersions. RMPC samples showed high UHT stability as compared to concentrated RSMPs at much higher protein levels. RMPC with 8% protein was very stable during UHT processing (data not shown), therefore the amount of protein in samples was increased further. 10-RMPC and 14-RMPC samples showed no signs of fouling throughout the run-time of 120 min and there was an insignificant drop in OHTC (Fig 3.4B). The ethanol stability of 14-RMPC (86%) was significantly higher than that of 7.5-RSMP (54%) (Table 3.2). Heat stability behaviour of milk protein dispersions when measured by HCT was also in agreement with their UHT stability. HCT for sample 7.5-RSMP (1.77 min) was low as compared to 14-RMPC (2.54 min) (Table 3.2). The UHT behavior of 14-RMPC-G prepared from MPC85 obtained from a different supplier showed similar UHT stability results. This was done to eliminate the possibilities of any differences between UHT stability of reconstituted samples prepared from MPC85 manufactured by different manufacturers.

Table 3.2: Calcium ion activity, ethanol stability, viscosity, HCT, lactose and major milk minerals of selected samples.

Sample	Calcium ion activity (mM)	Ethanol Stability (%)	Viscosity (mPa.s)		HCT (min)
	Unheated	Unheated	Unheated	UHT	
7.5-RSMP	1.36±0.01^{b}	54.00±0.00^{d}	6.79±0.64^{c}	12.97±1.66^{b}	1.77±0.16^{c}
14-RMPC	1.98±0.02^{a}	86.00±0.00^{b}	32.43±0.89^{a}	34.74±4.36^{a}	2.54±0.11^{b}
7.5-RMPC-LAC	2.00±0.13^{a}	88.00±0.00^{a}	8.85±0.49^{c}	5.50±0.03^{c}	2.82±0.16^{a}
7.5-RMPC-LAC-SMUF	1.30±0.02^{b}	59.00±0.89^{c}	19.20±2.82^{b}	7.53±0.46^{bc}	1.51±0.10^{d}

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different (P<0.05)

Unheated= unheated sample, UHT= UHT treated sample

3.3.3 Effect of total solids and minerals on UHT stability of milk protein dispersions

A distinguishing difference between RMPC and RSMP samples may be that RMPC samples had much lower TS (Table 3.1) than RSMP samples, which can lead to better heat stability under UHT conditions. High viscosities of concentrated samples can shift the fluid flow behaviour from turbulent to laminar; which can cause low flow rates for layers of process fluid adjacent to the heat transfer surface, resulting in a larger volume of material in contact with the heating surface for longer period of time. This can lead to the formation of a larger volume of fouling deposits (Burton, 1994). However, 14-RMPC showed high UHT stability even though its viscosity was significantly (P<0.05) higher than 7.5-RSMP (Table 3.2). It is important note that according to national dysphagia diet task force (NDDTF) of American dietetic association, USA, beverages can be classified as thin (1–50 mPas), nectar-like (51–350 mPa.s), honey-like (351–1750 mPa.s) and pudding-like (>1750 mPa.s) at the shear rate of swallowing (50 s-1) (NDDTF, 2002). According to this categorisation, the UHT processed samples studied in this work showed a thin liquid to nectar like behaviour.

Therefore in order to look into the effect of total solids on UHT heat stability of milk protein dispersions, lactose was added to 7.5-RMPC (7.5-RMPC-LAC) to match TS of 7.5-RSMP. 7.5-RMPC-LAC showed a UHT run time of greater than 120 min (Fig 3.3). Also, the OHTC over the run time of 7.5-RMPC-LAC was similar to 14-RMPC and much higher than 7.5-SMP (Fig 3.4C). Ethanol stability and HCT of 7.5-RMPC-LAC were significantly (P<0.05) higher than that of 7.5-SMP (Table 3.2) and were similar to that of 14-RMPC. Hence, it could be concluded that 7.5-RMPC-LAC had

much higher UHT stability than 7.5-SMP at same TS content and could be processed without fouling. The results suggested that milk constituents other than proteins, such as milk minerals (in particular calcium), may be responsible for the differences in UHT stability and susceptibility to fouling of RMPCs and RSMPs. High calcium ion activity has been associated with decreased UHT stability of milk products (Singh, 2004). However, 14-RMPC had significantly ($P<0.05$) higher ionic calcium activity as compared to 7.5-RSMP (Table 3.2), which does not correlate with UHT stability of these two high protein samples. Hence, calcium ion activity alone could not be the dominating factor explaining the differences between the UHT stability of these samples. Similar results on ionic calcium and heat stability behaviour of milk protein concentrate suspensions (MPC80) was reported by Crowley et al. (2015) .

To investigate the synergetic effect of milk proteins, lactose and milk minerals on UHT stability of RMPC, an MPC dispersion (7.5-RMPC-LAC-SMUF) containing the same amount of proteins, lactose and mineral salts as 7.5-RSMP was prepared. The amount of SMUF used in 7.5-RMPC-LAC-SMUF was calculated on the basis of matching total calcium of this sample to 7.5-RSMP, which also closely matched the amount of lactose and other milk minerals such as magnesium, phosphorous etc. in these two samples (Table 3.3). The calcium ion activity of 7.5-RMPC-LAC-SMUF was found to be 1.30 mM, which was very close to that of 7.5-RSMP (1.36 mM). During UHT processing, 7.5-RMPC-LAC-SMUF showed 66 min run-time on average (Fig 3.3). The sample showed an induction time of 21 min, after which frequent temperature and back pressure fluctuations were observed (Fig 3.4C) and the UHT run had to be terminated after 66 min due to fouling. The induction period of 21 min showed by 7.5-RMPC-LAC-SMUF was very close to total run-time of 23 min observed for 7.5-RSMP.

Table 3.3: Lactose and major milk minerals present in selected samples.

Sample	Lactose (% w/w)	Calcium (% w/w)	Phosphorous (% w/w)	Magnesium (% w/w)	Chloride (% w/w)	Potassium (% w/w)	Sodium (% w/w)
7.5-RSMP	12.69	0.34	0.24	0.03	0.14	0.30	0.10
14-RMPC	0.86	0.38	0.25	0.02	0.03	0.06	0.02
7.5-RMPC-LAC	14.73	0.20	0.14	0.01	0.01	0.03	0.01
7.5-RMPC-LAC-SMUF	12.96	0.36	0.28	0.04	0.35	0.58	0.21

Values derived from proximate and mineral composition of milk powders used to prepare the samples, except for sample 7.5-RMPC-LAC-SMUF values measured using ICP-OES.

Ethanol stability and HCT of RMPC reduced markedly after addition of milk minerals; 7.5-RMPC-LAC-SMUF showed ethanol stability (59%) and HCT (1.51 min) similar to 7.5-SMP (Table 3.2). The study conducted by Crowley et al. (2015) on heat stability behaviour of RMPCs containing 8.5% protein also showed that HCT of reconstituted MPC35 (composition closely matching to an SMP) at pH 6.8 was lower than that of reconstituted MPC80; however, UHT stability was not studied. Milk ethanol stability values of 74% and above are considered suitable for UHT processing (Deeth & Lewis, 2017; Shew, 1981). The ethanol stability of milk protein dispersions (Table 3.2) and their UHT stability (Figure 3.3) were in agreement of this recommendation. The ethanol stability test is used to determine casein micelle stability. Ethanol collapses the κ-casein hairy layer on the casein micelle surface and its function of steric stabilization is lost, leading to casein micelles coagulation (Horne, 1984). The ethanol stability results were similar to UHT stability of milk protein dispersions studied.

3.3.4 Particle size distribution of milk protein dispersions

PSD data (performed on the whole sample) of UHT processed samples demonstrated the formation of larger size particles in 7.5-RSMP as compared to 14-RMPC (Fig 3.5). Almost all the particles in UHT processed 14-RMPC were of sub-micron size (0.23 μm D(0.9)) as compared to 7.5-RSMP (1.84 μm D(0.9)) as shown in Table 3.4. These differences in particle size were significant and were possibly due to the differences the mineral environment of the samples. The large particles could be formed from whey protein interactions amongst themselves and or with caseins to for whey protein-casein aggregates. The differences in the mineral environment can affect the size of whey protein aggregates formed during UHT treatment and can lead to the formation of larger aggregates in case of RSMP and RMPC-LAC-SMUF (Crowley et al., 2015; Havea, Singh, & Creamer, 2002).

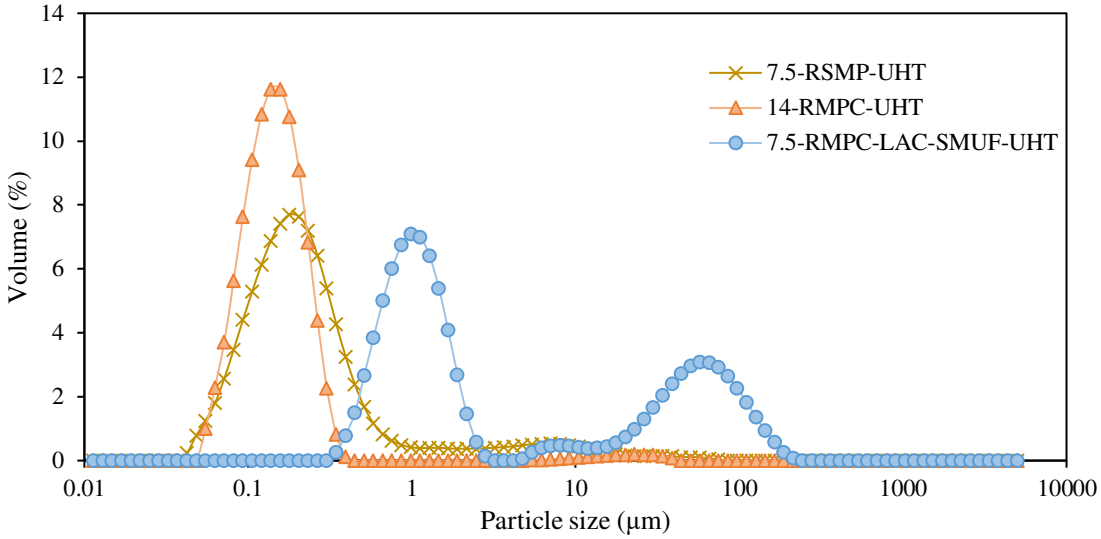

Figure 3.5: Particle size distribution of UHT processed milk protein dispersions. Average data of four measurements.

Table 3.4: Comparison of volume weighted mean diameter, Surface weighted mean diameter and particle size distribution for 7.5RSMP and 14-RSMPC.

Sample	D[4,3] (µm)	D[3,2] (µm)	D(0.1) (µm)	D(0.5) (µm)	D(0.9) (µm)
7.5-RSMP	1.12±0.50	0.16±0.01	0.08±0.00	0.19±0.01	1.84±1.38
14-RMPC	0.50±±0.17	0.12±±0.00	0.08±±0.00	0.13±0.00	0.23±0.00
7.5-RMPC-LAC-SMUF	22.43±2.99	1.40±0.30	0.62±0.10	1.39±0.29	74.71±4.49

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different (P<0.05).

3.3.5 Protein profile of milk protein dispersions

We further investigated the effect of mineral environment on protein dissociation in samples before and after UHT processing. RP-HPLC analysis was performed on supernatants of unheated and UHT processed 7.5-RSMP, 14-RMPC and 7.5-RMPC-LAC-SMUF samples (Fig 3.6). The RP-HPLC data showed that in all three samples, β-lg was completely aggregated after UHT processing (Fig 3.6A) and was absent from the non-sedimentable fraction. Additionally more than 75% of α-la was aggregated in all three samples (Fig 3.6B). Crowley et al. (2015) also showed that the difference in the amount of non-sedimentable whey proteins in heated reconstituted MPC80 and MPC35 was not significant. But it is possible that the types of aggregates formed from these non-sedimentable whey proteins upon heating are responsible for differences in UHT stability of RMPC and RSMP samples as described above.

Significant differences were observed in the non-sedimentable caseins between 14-RMPC, 7.5-RSMP-UHT and 7.5-RMPC-LAC-SUMF before and after UHT treatment. This is interesting because the stability of casein micelles during UHT processing could be another factor governing UHT stability of RMPC samples. It was observed from RP-HPLC data that unheated samples of 14-RMPC had significantly ($P<0.05$) higher amounts of dissociated caseins as compared to 7.5-RSMP and 7.5-RMPC-LAC-SMUF (Fig 3.6C-E). Non-sedimentable protein content in all three samples was similar after UHT processing, however, when comparing unheated samples to UHT treated samples, it slightly increased in 7.5-RSMP and significantly ($P<0.05$) increased 7.5-RMPC-LAC-SMUF after UHT processing whereas it significantly ($P<0.05$) decreased in 14-RMPC (Fig 3.6F).

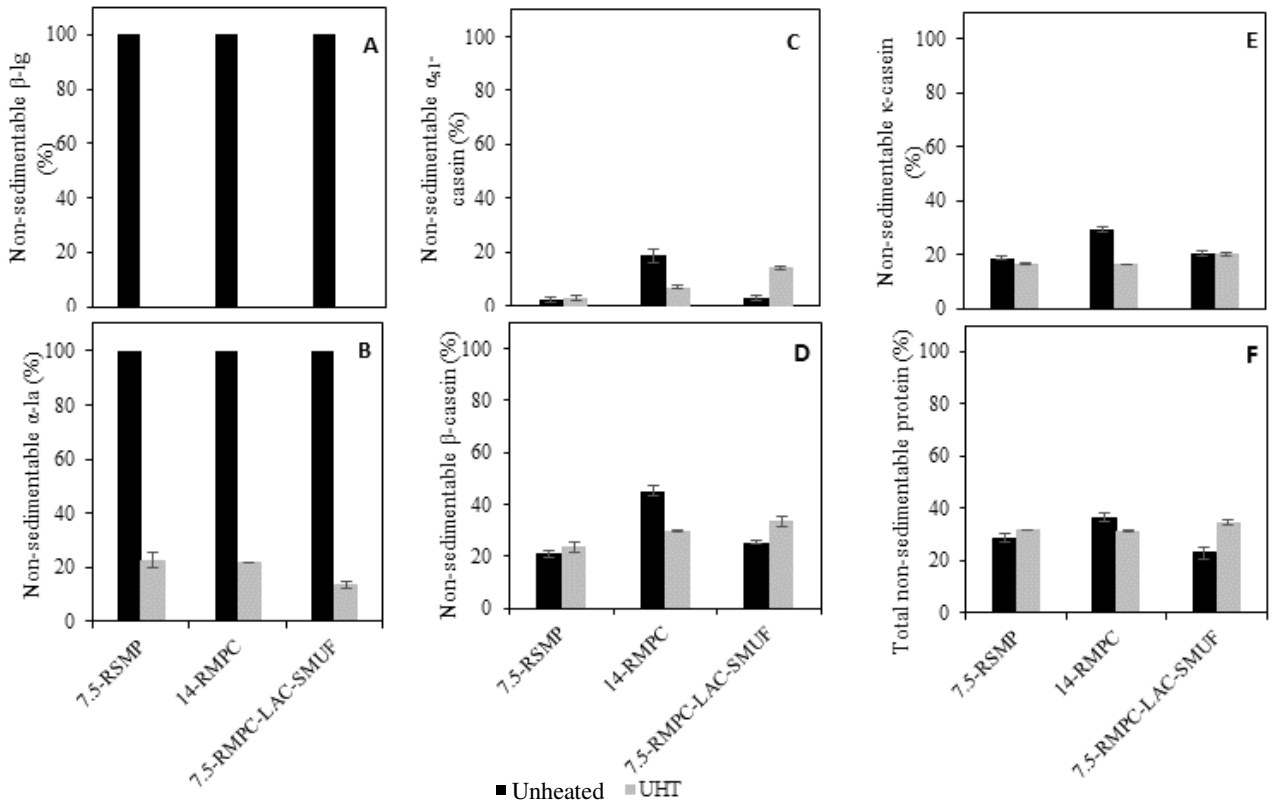

Figure 3.6: Effect of UHT processing of milk protein dispersions on non-sedimentable milk proteins (data shown as percentage of non-sedimentable protein of individual and total milk protein present in the sample). (A) β-lg , (B) α-la, (C) αs1-casein, (D) β-casein (E) κ-casein and (F) Total non-sedimentable protein. Error bars represent standard deviation, n=2. Unheated= unheated sample, UHT= UHT treated sample.

This may suggest that casein micelles in 7.5-RSMP and 7.5-RMPC-LAC-SUMF were more unstable to UHT treatment than in 14-RMPC. It could be possible that in 14-RMPC the initially dissociated caseins might have deposited on heated surfaces initially (Santos et al., 2003), but there was not much further dissociation of caseins during UHT processing to participate in the formation of large aggregates. In 7.5-RSMP higher dissociation of casein micelles during UHT treatment might have happened, bringing its post UHT non-sedimentable protein content almost similar to heated 14-RMPC. These UHT induced dissociated caseins might have led to the formation of large aggregates due to whey-casein aggregation via κ-casein-β-lg interactions or aggregation of unstable casein micelles (Anema & Li, 2003b; Ono et al., 1999). Smaller protein aggregates formation observed in UHT processing of MPC could be related to its altered mineral environment during manufacturing (Crowley et al., 2015).

The ethanol stability data (Table 3.2) also showed that 14-RMPC had higher ethanol stability than 7.5-RSMP, implying higher casein micelle stability in 14-RMPC. As RP-HPLC results (Fig 3.6E) showed that κ-casein content in all three UHT processed samples was similar it can be concluded that either electrostatic interactions between caseins or extent of collapse of κ-casein hairy layer and loss of steric stabilization during UHT processing of these protein dispersions were influenced by soluble salts in the serum phase (Horne, 2016). As stated above, the mineral environment of RSMP appeared to be favourable to start rapid interactions of casein micelles as observed by Horne (1984); Horne and Parker (1981) and indicated by low ethanol stability shown by RMPC with added SMUF (7.5-RMPC-LAC-SMUF).

It was observed that milk proteins behaved differently in different mineral environments. Milk protein dispersions prepared from RMPC formed submicron particles after UHT treatment as shown by PSD and dissociation of caseins was limited in the RMPC mineral environment as compared to RSMP as shown by RP-HPLC and ethanol stability data. The similarity in UHT behaviour of 7.5-RSMP and 7.5-RMPC-LAC-SMUF and drop in UHT stability of RMPC after addition of minerals was an indicator that total milk mineral environment plays a crucial role in UHT stability of high protein dispersions. Effect of UHT temperatures on milk protein stability, changes in protein state and their interactions with milk minerals during heating of milk protein dispersions prepared from these two different milk protein powders can be an important factor in determining their fouling behaviour. This suggest that difference in mineral composition of MPC powder from SMP due to ultrafiltration process can be an important factor causing its high heat stability. During SMP manufacturing all the milk minerals are retained in the final product, however during membrane filtration process employed during the manufacturing of MPC, free ions pass through the membrane

and protein is retained, which increases the volume fraction of caseins and changes ratio between soluble and colloidal minerals (Dalgleish & Corredig, 2012). This also alters the casein inter-micelle interactions. Mineral composition of aqueous phase has also been found to have a significant role on physicochemical properties and heat stability of reconstituted casein micelles (Le Ray et al., 1998). The higher instability of 7.5-RMPC-LAC-SMUF compared to 7.5-SMP is being further investigated.

3.4 Conclusions

MPC is an important ingredient of milk protein based beverages, however, there is little known about their UHT stability. MPC reconstituted to 14% protein showed significantly higher UHT stability as compared to SMP reconstituted at 7.5% protein, although the ionic calcium activity and viscosity of the former was higher than the later. The lower UHT stability of RSMP can be related to larger protein aggregate formation and destabilization of casein micelles in 7.5-RSMP at UHT temperatures. High UHT stability of milk protein dispersions made from high protein milk powder, such as MPC85, can be due to the ultrafiltration processing used during their manufacturing, which causes them to have a modified mineral composition as compared to SMP. The UHT instability of mineral readjusted MPC85 even at 7.5% protein concentration suggested that the total mineral composition is responsible for fouling of high protein SMP suspension. Further investigation is underway to explore the effect of changes in mineral composition on UHT behaviour of MPC powders at different protein concentrations.

CHAPTER 4: EFFECT OF ADDITION OF MINERAL SALTS ON ULTRA-HIGH TEMPERATURE STABILITY OF RECONSTITUTED MILK PROTEIN CONCENTRATE.

The following submitted manuscript has been incorporated as Chapter 4:

Singh, J., Dean A, Prakash, S., Bhandari, B., & Bansal, N. (Submitted in March, 2020). The role of minerals in higher ultra-high temperature stability of milk protein concentrate compared to conventional low heat skimmed milk powder, under review in Journal of Food Engineering.

Contributor	**Statement of contribution**
Jaspal Singh (Candidate)	Concept and design (80%) Analysis and interpretation (60%) Drafting and production (55%)
Agathe Dean	Concept and design (5%) Analysis and interpretation (20%) Drafting and production (10%)
Sangeeta Prakash	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (5%)
Bhesh Bhandari	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (5%)
Nidhi Bansal	Concept and design (5%) Analysis and interpretation (10%) Drafting and production (25%)

Abstract: The role of mineral salts in heat stability of reconstituted milk protein concentrate (RMPC) as compared to reconstituted skim milk powder (RSMP) was studied. 7.5% protein RMPC showed higher thermal stability than 7.5% protein RSMP; their heat coagulation time was 6.80 and 1.80 min, respectively. Matching the total mineral balance of 7.5-RMPC with 7.5-RSMP decreased its HCT to 1.55 min. Amounts of individual minerals, Ca, Mg, Na and K, of RMPC were matched with RSMP using mineral salts. $CaCl_2$ added 7.5-RMPC sample could not be UHT processed due to its low heat stability. Addition of NaCl to protein RMPC did not adversely affect their UHT stability (UHT run-time >120 min). KCl added samples showed diminished UHT stability (run-time 15 min) and drop in overall heat-transfer coefficient. High apparent viscosity and formation of larger particles were observed in this sample as compared to in RMPC. It was found that overall mineral balance with a reduced amount of minerals per protein unit are responsible for high heat stability for RMPC compared to RSMP.

4.1 Introduction

The mineral fraction makes up to just 0.8-0.9% of total milk constituents, which primarily contains calcium, inorganic phosphorous, magnesium, sodium, potassium, citrate and chlorine (Gaucheron, 2005). However, milk minerals play an important role in governing the heat stability of milk and milk products (Horne & Muir, 1990). Calcium phosphate and calcium citrate are the main salts presents in milk (De la Fuente, 1998). Calcium exists in milk partly associated with casein micelles (colloidal calcium phosphate) and partly in the serum phase as free calcium ions and calcium Citrate anions (Walstra et al., 2005). Calcium has been long recognized as influencing the casein micelle colloidal stability in milk, where ionic calcium levels are also a function of milk pH (Jeurnink & De Kruif, 1995; Singh, 2004). A decrease in pH or addition of calcium salts leads to increase in ionic calcium levels in the serum. This is particularly important during heat treatment as higher levels of ionic calcium in the serum environment have been linked with diminished heat stability of milk and milk products by reducing repulsions between negatively charged casein micelles and promoting inter-micellar interactions (Crowley, Kelly, & O'Mahony, 2014; Kastanas, 1996). Adding phosphorous and Citrate to milk can chelate calcium ions and enhance its heat stability (O'Connell & Fox, 2011). However, casein micelles are swollen in milk with reduced calcium content and a large amount of β- and κ-casein are depleted from micelles causing them to destabilize (Jeurnink & De Kruif, 1995). Magnesium and calcium are shown to increase the aggregation of denatured whey proteins in heated whey protein isolate solutions (Varunsatian, Watanabe, Hayakawa, & Nakamura, 1983; Xiong, 1992).

The mineral environment of milk system can also affect the size of denatured whey proteins aggregates as shown by Crowley et al. (2015), when comparing heat stability of low and high protein milk protein concentrates (MPC). Addition of NaCl to milk increases the soluble calcium levels, reduces the net negative charge on casein micelles and causes a shift in the pH range of maximum coagulation time (HCT) in HCT-pH profile (Huppertz & Fox, 2006). The mineral balance in milk affects the stability of milk proteins due to the tendency of salts to change hydrophilic-hydrophobic characteristics of milk proteins and ionic strength (Mao, Tong, Gualco, & Vink, 2012). Additionally, milk salts such as calcium phosphate tend to precipitate during processing due to physico-chemical reactions leading to their reduced solubility at high temperatures as observed in UHT treatment (Visser & Jeurnink, 1997).

Milk salts naturally remain in an equilibrium between micellar and serum phase of milk until this equilibrium is altered by some processing techniques (De la Fuente, 1998). MPC is a form of milk powder, which contains an altered mineral composition as compared to skimmed milk powder (SMP)

due to membrane processing used in its manufacturing, where some minerals are partly depleted in the supernatant (Sikand, Tong, Roy, Rodriguez-Saona, & Murray, 2011). In general, MPC contains higher amounts of calcium and phosphorus but lower amounts of sodium and potassium as compared to SMP (Augustin et al., 2011; Zwijgers, 1992). However, MPC contains lower amounts of calcium and phosphorous per unit protein as compared to SMP, when reconstituted, as shown by Crowley, Megemont, et al. (2014) and Chapter 3.

MPC contains casein and whey proteins largely in their native state and composition as found in milk. The protein content of MPC can range from 35 to 90%, which makes them a very good source of protein (Meletharayil, Patel, Metzger, & Huppertz, 2016). MPC is commonly used as a concentrated protein ingredient in protein beverages and imparts nutritional, functional and sensory properties to the product (Gandhi, Amamcharla, & Boyle, 2017). High protein beverages are required to be thermally processed before packaging. There are many factors affecting the heat stability of milk products and mineral balance is one of them. The modified mineral environment of MPC can modifiy its heat stability behavior compared to conventional SMP, which was shown by our work on UHT stability of MPC dispersions. Chapter 3 results indicated that altered mineral composition of MPC could be responsible for its high UHT stability. Reconsituted MPC (RMPC) showed much higher heat stability than reconsituted SMP (RSMP) even in the case where RMPC was UHT processed at almost double the protein concentration of RSMP.

The present study focuses on further investigating the role of altered mineral composition of MPC in its heat stability. The influence of matching the minerals of MPC to that of SMP on the heat stability of MPC are studied in this work, while keeping other factors constant.

4.2 Materials and methods

4.2.1 Materials

Commercially manufactured MPC and low heat SMP used for the preparation of all milk protein dispersions were supplied by Maxum Foods Pty. Ltd, Australia. All chemicals and reagents used in this study were purchased from Sigma-Aldrich Pty. Ltd., NSW, Australia unless otherwise stated. Recipe for simulated milk ultrafiltrate (SMUF) was adopted from Jenness (1962). The mineral and compositional analysis of MPC and SMP was carried out as described in Chapter 3. MPC and SMP contained on average 81.5 and 32.5% total protein, respectively.

4.2.2 Preparation of reconstituted milk protein dispersions

Milk proteins dispersions were prepared at required protein content by dispersing calculated amounts of milk powders and other ingredients. Table 4.1 gives a description of different samples used in this study. Samples were prepared by using the method described in Chapter 3. The pH of protein dispersions were analysed and adjusted to 6.8, if required. All the samples were filtered to remove any undissolved particles (almost negligible).

Table 4.1: Description of reconstituted milk protein dispersions used.

Sample	Ingredients	Description
7.5-RSMP	Reconstituted skim milk powder (RSMP)	7.5% protein content
7.5-RMPC	Reconstituted milk protein concentrate (RMPC)	7.5% protein content
7.5-RMPC-SMUF	MPC, lactose and SMUF	7.5-RMPC sample prepared to match its total solids and mineral composition to 7.5-RSMP using SMUF
7.5-RMPC-Ca	MPC and $CaCl_2.2H_2O$	7.5-RMPC samples prepared to match their targeted minerals with 7.5-RSMP.
7.5-RMPC- Mg	MPC and $MgCl_2.6H_2O$	
7.5-RMPC-Na	MPC and NaCl	
7.5-RMPC-K	MPC and KCl	

4.2.3 Ionic Ca activity in protein dispersions

Ionic Ca in samples was measured using a LAQUAtwin calcium ion meter (Horiba Instruments, Japan) as described in Chapter 3. All measurements were performed at room temperature.

4.2.4 Heat coagulation time measurements

The heat coagulation time (HCT) was used to assess heat stability of samples as per the method described by Davies and White (1966) with some modifications. The HCT was measured at a temperature similar to UHT sterilization (145 ºC). Glass vials (22.6 x 75.5 mm) containing 2 mL sample were placed on a rocker and immersed in a temperature controlled oil bath for heating. The rocker speed was kept at ~8 revolutions per min. HCT was measured as time the elapsed between

putting the samples in the oil bath and appearance of first visible signs of coagulation. All experiments were performed in triplicate.

4.2.5. UHT stability and fouling measurements

A bench-top UHT plant was used for UHT processing of samples. A complete description and flow diagram of the plant are given in Chapter 3. The sample pre-heating temperature was 95 °C for 8 s before UHT processing them at 145 °C for 5 s. The product volumetric flow rate in the UHT plant processing line at the beginning of the experiment was kept at 150 mL/min ($2.5X10^{-6}$ m^3/s). Duplicate UHT runs were performed and the results presented are an average of the two runs. The fouling indicators used to detect signs of fouling were as follows: (i) The back pressure could not be maintained at 0.4 MPa and high back pressure triggered the over pressure valve, (ii) the outlet temperature of the sterilization section dropped below 120 ºC and (iii) in some cases, blockage of product channel due to severe fouling. Unless otherwise stated, if none of the above factors stopped UHT processing, the experiment was terminated after 120 min has elapsed into the UHT run. Fouling behaviour and UHT stability of samples was monitored using the drop in overall heat transfer co-efficient (OHTC) during UHT run as described in Chapter 3.

4.2.6 Particle size distribution of protein dispersions

Particle size distribution (PSD) of samples was analysed by dynamic light scattering (DLS) method using Malvern Mastersizer 2000MU-A (Malvern Instruments Ltd, Malvern, United Kingdom) as described by (Dumpler & Kulozik, 2015). The refractive index of protein and dispersant (distilled water) was set at 1.41 and 1.33, respectively. Particle absorption index was kept as 0.001. Stirrer speed was set at 2000 rpm and laser obscuration was maintained between 10 and 11 during measurement. All measurements were performed at room temperature (22-23 °C). All experiments were performed in duplicate.

4.2.7 Viscosity measurements

The apparent viscosity of milk protein dispersions was measured at a shear rate of 300 s^{-1} and 20 ºC using AR-G2 Rheometer (TA Instruments Ltd., USA) equipped with a 60 mm parallel plate. A 300 µm gap was set to place sample between the upper plate and pettier plate. Measurements were performed on samples after 1 min of temperature equilibration. All experiments were performed in duplicate.

4.2.8 Non-sedimentable protein analysis

The ultracentrifuged (100,000 *g* for 1 h at 20 °C using Avanti JXN-30, Beckman Coulter, Australia Pty. Ltd., NWS, Australia) supernatants of unheated and UHT processed samples were analysed for

non-sedimentable proteins using Kjeldahl method (AOAC, 2005). Individual non-sedimentable proteins of interest in supernatants were quantified using Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) (RP-HPLC-UV, Agilent 1100, Agilent Technologies Australia, Victoria, Australia) using method described by Wijayanti et al. (2013) and Chapter 3. A Zorbax 300SB-C18 column (300Å, Size: 150 x 4.60 mm I.D., Agilant Australia Pty Ltd.) equipped with a stainless steel Zorbax HPLC guard column (C18 4 x 12.5 mm, Agilant Australia Pty Ltd) was used. Duplicate analyses for each sample were performed.

4.2.9 Statistical analysis

The data was analysed using Microsoft Excel and Minitab 16 software package. Significant differences between average values of replicate measurements on each data point was analysed by analysis of variance (ANOVA) using Tukey's HSD post hoc test at 95% confidence level.

4.3 Results and Discussion

4.3.1 Effect of minerals on HCT of milk protein dispersions

Mineral analysis of SMP and MPC showed a large difference in their major minerals composition that resulted in a very different mineral environment when these were reconstituted at similar protein content (Table 4.2). 7.5-RSMP contained all the major milk minerals in higher proportions than 7.5-RMPC. In order to study the effect of added minerals on the heat stability of milk protein dispersions, SMUF and mineral salts were added to 7.5-RMPC and their mineral composition is presented in Table 4.2.

Table 4.2: Major milk minerals in milk protein dispersions (% w/w)

Sample	Ca	P	Mg	Cl	K	Na
7.5-RSMP	0.34	0.24	0.03	0.14	0.30	0.10
7.5-RMPC	0.22	0.13	0.01	0.01	0.04	0.01
7.5-RMPC-SMUF	0.35	0.28	0.04	0.35	0.58	0.21
7.5-RMPC-Ca	0.36	0.15	0.01	0.25	0.04	0.01
7.5-RMPC- Mg	0.23	0.14	0.04	0.08	0.04	0.01
7.5-RMPC-Na	0.22	0.14	0.01	0.20	0.04	0.09
7.5-RMPC-K	0.22	0.14	0.01	0.25	0.29	0.01

Mineral values measured using ICP-OES.

The effect of adding various minerals to RMPC on ionic calcium and HCT was measured in comparison with 7.5-RSMP and 7.5-RMPC and is shown in Figure 4.1 and 4.2, respectively. The total calcium content of 7.5-RSMP was higher than 7.5-RMPC, however the ionic calcium of 7.5-RMPC was higher than 7.5-RSMP (2.12 and 1.35 mM, respectively, Fig 4.1). When SMP is reconstituted in water the calcium in colloidal and serum phase remains in equilibrium similar to milk and calcium stays as a stable complex formed largely with citrate and to a lesser extent with inorganic phosphate and with chloride (Gaucheron, 2005). However, in case of 7.5-RMPC the ionic calcium remained high due to the fact that most of the other soluble minerals such as citrate, phosphate and chloride in the serum phase are removed during membrane filtration of MPC. This leads to higher levels of free ionic calcium in 7.5-RMPC.

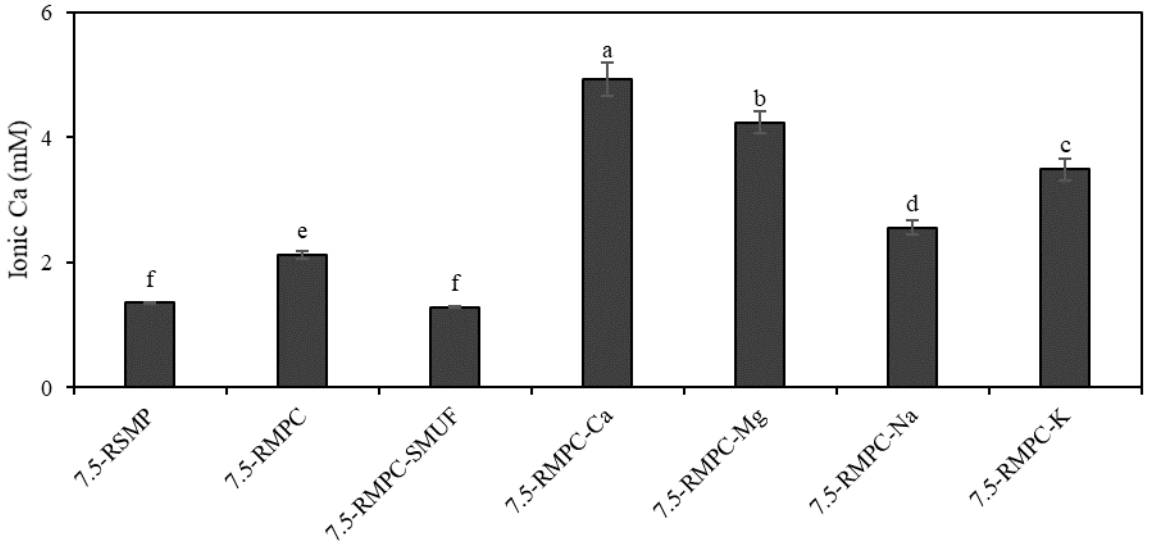

Figure 4.1: Ionic calcium in unheated milk protein dispersions. Error bars represent standard deviation, n=3. Means with different letters are significantly different (P < 0.05).

High ionic calcium in milk products is often considered detrimental to their stability during thermal processing (Karlsson et al., 2019; Lewis, Grandison, Lin, & Tsioulpas, 2011; Singh, 2004). However, 7.5-RMPC showed significantly higher heat stability than 7.5-RSMP (HCT values of 6.80 and 1.80 min, respectively) (Fig 4.2). Similar results on ionic calcium and HCT of concentrated MPC dispersions were reported by Crowley et al. (2015). Which points out that factors other than ionic calcium could be responsible for high heat stability of 7.5-RMPC.

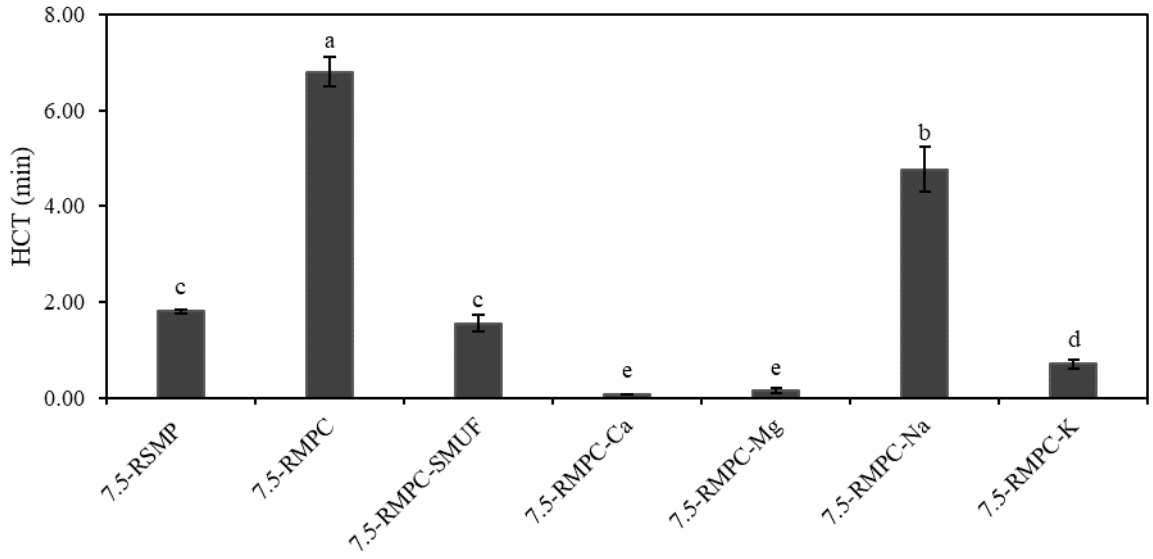

Figure 4.2: HCT of milk protein dispersions at 145 °C. Error bars represent standard deviation, n=3. Means with different letters are significantly different (P < 0.05).

The effect of other milk minerals on heat stability of 7.5-RMPC was studied by addition of SMUF to it (7.5-RMPC-SMUF). The SMUF amount required for this purpose was calculated on the basis of matching total calcium of this sample to 7.5-RSMP, which also closely matched the amount of other milk minerals such as magnesium, phosphorous etc. and ionic calcium in these two samples (Table 4.2 and Fig 4.1). The HCT of 7.5-RMPC decreased after addition of SMUF and HCT of 7.5-RMPC-SMUF was very similar to 7.5-RSMP (Fig. 4.2). This further indicated towards the role of mineral composition of 7.5-RMPC altered by membrane filtration in its high heat stability.

The effect of addition of individual major milk minerals on heat stability of 7.5-RMPC was further investigated. 7.5-RSMP contained higher amounts of cationic minerals as compared to 7.5-RMPC (Table 4.2). The addition of different mineral salts can change the ionic strength of milk protein dispersion depending upon the amount of mineral salts added. Divalent and monovalent cations are considered to be responsible for reduction of electrostatic and steric repulsion in milk proteins leading to protein coagulation (Karlsson, Ipsen, Schrader, & Ardö, 2005). This can result in poor stability of milk product during heating. Increasing the concentration of calcium and magnesium in 7.5-RMPC (7.5-RMPC-Ca and 7.5-RMPC-Mg, respectively) to the levels of 7.5-RSMP caused increase in ionic calcium of these samples (Fig. 4.1). The heat stability of these samples was extremely poor (Fig. 4.2). Similarly, a reduction in heat stability of casein micelles dispersions after addition of cationic salts was reported by Philippe, Le Graët, and Gaucheron (2005). Addition of NaCl and KCl to 7.5-RMPC caused an increase in ionic calcium levels on resulting protein dispersions (7.5-RMPC-Na and 7.5-RMPC-K), but to a lower extent than 7.5-RMPC-Ca and 7.5-RMPC-Mg (Fig. 4.1). The increase in

ionic calcium after salt addition can be attributed to the fact that some of the calcium of the casein micelles was replaced by sodium, potassium or magnesium ions releasing calcium ions into the serum phase (Sikand, Tong, & Walker, 2013). The increase in ionic strength in RMPC induced by NaCl and KCl could result in a reduction in the ion activity coefficeint of calcium phosphate increasing its solubility (Huppertz & Fox, 2006), however it has been reported that increase in ionic calcium after NaCl addition was due to realease of calcium bound to caseins rather than solubilisation of calcium-phosphate nano clusters (Dalgleish & Parker, 1980; Parker & Dalgleish, 1981; Singh & Fox, 1987).

The HCT of 7.5-RMPC also decreased significantly after an increase in its monovalent ions concentrations, however their HCT still remained significantly higher than 7.5-RMPC-Ca and 7.5-RMPC-Mg (Fig. 4.2). This can be due to the ability of divalent ions to destabilize casein micelle colloidal stability more efficiently than monovalent ions (Keowmaneechai & McClements, 2002). Addition of KCl lowered the HCT of 7.5-RMPC much more significantly than NaCl. This difference in HCT behaviour of 7.5-RMPC with increased monovalent ions concentrations could be related to the higher amount of KCl required to increase potassium content in 7.5-RMPC as compared to NaCl amount required to increase the sodium content. In general, an increase in potassium and sodium content in milk and milk protein products beyond a certain level had been shown to negatively affect their heat stability due to reduced net negative charge on casein micelles and coagulation of proteins. (Guo et al., 1998; Hunt & Dalgleish, 1995; Huppertz & Fox, 2006; Walstra & Jenness, 1984). Increase in milk potassium content by the addition of KCl is considered to affect milk heat stability to a greater extent as compared to NaCl addition due to higher cation exchange efficiency of potassium (Morrissey, 1969). This is due to the fact that potassium is a more reactive alkali metal than sodium. Both have one valence electron in their outermost shell however potassium has four shells as compared to three in sodium. This makes potassium to lose its valence electron easier than sodium due to the greater distance and weaker pull from its nucleus (Blackman, 2018).

4.3.2 Effect of minerals on UHT stability of milk protein dispersions

HCT results showed the importance of concentration of major divalent and monovalent cations in determining the heat stability of RMPC. For further heat stability analysis, UHT processing of milk protein dispersions was carried out. The fouling behaviour of mineral salt-added samples was analysed along with their physico-chemical properties to gain more insights into the effect of minerals on the heat stability of milk protein dispersions. From all the samples studied in HCT analysis, 7.5-RMPC, 7.5-RMPC-K and 7.5-RMPC-Na were selected for UHT analysis. Samples with added $CaCl_2$ and $MgCl_2$ were not selected for further analysis due to their extremely low HCT (a UHT trial on 7.5-

RMPC-Ca resulted in immediate blockage of UHT plant, data not shown). UHT stability of these samples followed a similar trend as shown by HCT analysis. 7.5-RMPC and 7.5-RMPC-Na were very stable during UHT processing and average run-times of bench-top UHT plant exceeded 120 min (Fig 4.3). These samples were UHT processed without any major temperature drops or pressure fluctuations and the UHT temperature was maintained at 145 ± 2 °C. However, 7.5-RMPC-K showed diminished UHT stability. The UHT run lasted for 15 min before it was stopped due to excessive fouling. Excessive temperature fluctuations were observed for this sample and development of excessive backpressure triggered the over pressure valve and the milk was pumped back into the balance tank of the UHT plant indicating its low UHT stability.

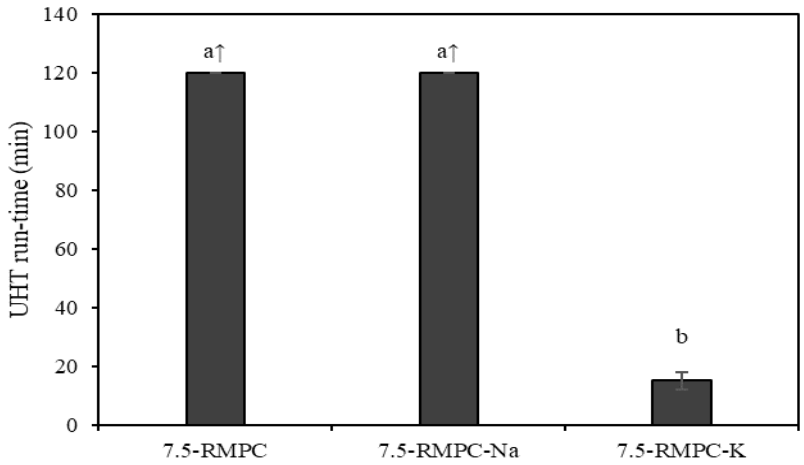

Figure 4.3: The average UHT run times on a bench top UHT tubular heat exchanger during processing of milk protein dispersions. Error bars represent standard deviation, n = 2. Means with different letters are significantly different (P < 0.05). Samples with ↑ did not foul in 120 min

The OHTC vs run-time graph (Fig 4.4) showed that the OHTC remained almost constant for 7.5-RMPC throughout the run. From minerals added samples, 7.5-RMPC-Na showed similar OHTC values as RMPC. However, during the UHT processing of 7.5-RMPC-K, there was a continuous decline in OHTC during the UHT run. The decrease in OHTC was due to the gradual fouling of heat transfer surfaces with milk solids, which caused increased resistance to heat transfer as compared to clean surfaces, decreasing UHT temperatures (Datta & Deeth, 2007).

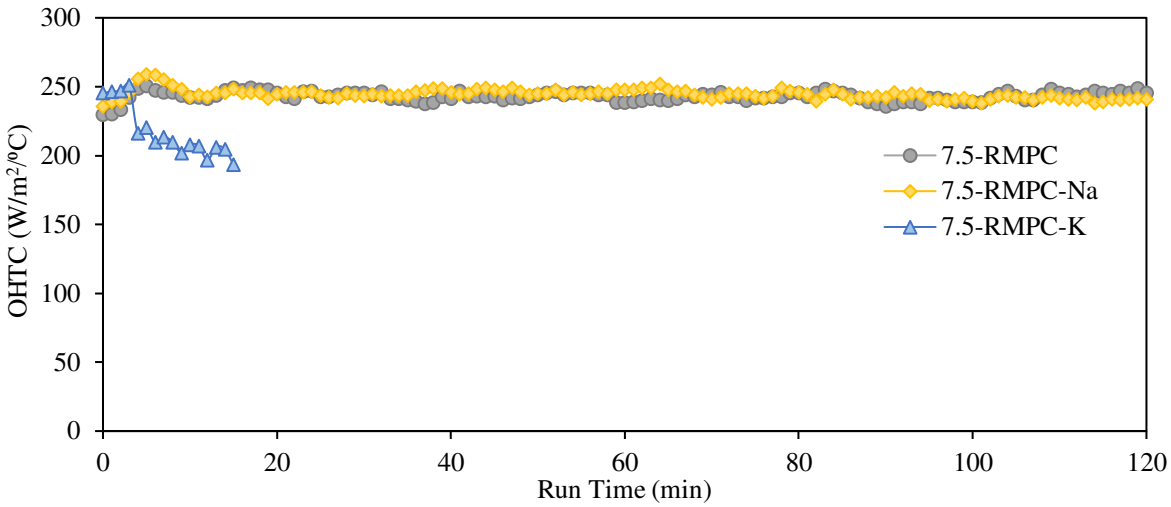

Figure 4.4: Variation in OHTC with run time for milk protein dispersions. Average data of duplicate runs is presented.

4.3.3 Effect of minerals on physico-chemical properties of milk protein dispersions

4.3.3.1 Particle size distribution

The PSD analysis on UHT processed milk protein dispersions provided further details about the effect of UHT processing on the aggregate formation of milk proteins in mineral-added samples as compared to 7.5-RMPC (Fig 4.5 and Table 4.3). Addition of minerals did not affect the particle size distribution of unheated 7.5-RMPC. Almost all the particles in all three unheated samples and UHT processed 7.5-RMPC were of sub-micron size. Most of the UHT processed 7.5-RMPC-Na particles were in the sub-micron range almost similar to UHT processed 7.5-RMPC but also showed a small second peak. Whereas, the particles in UHT processed 7.5-RMPC-K were almost entirely in the micron range suggesting the formation of large protein aggregates.

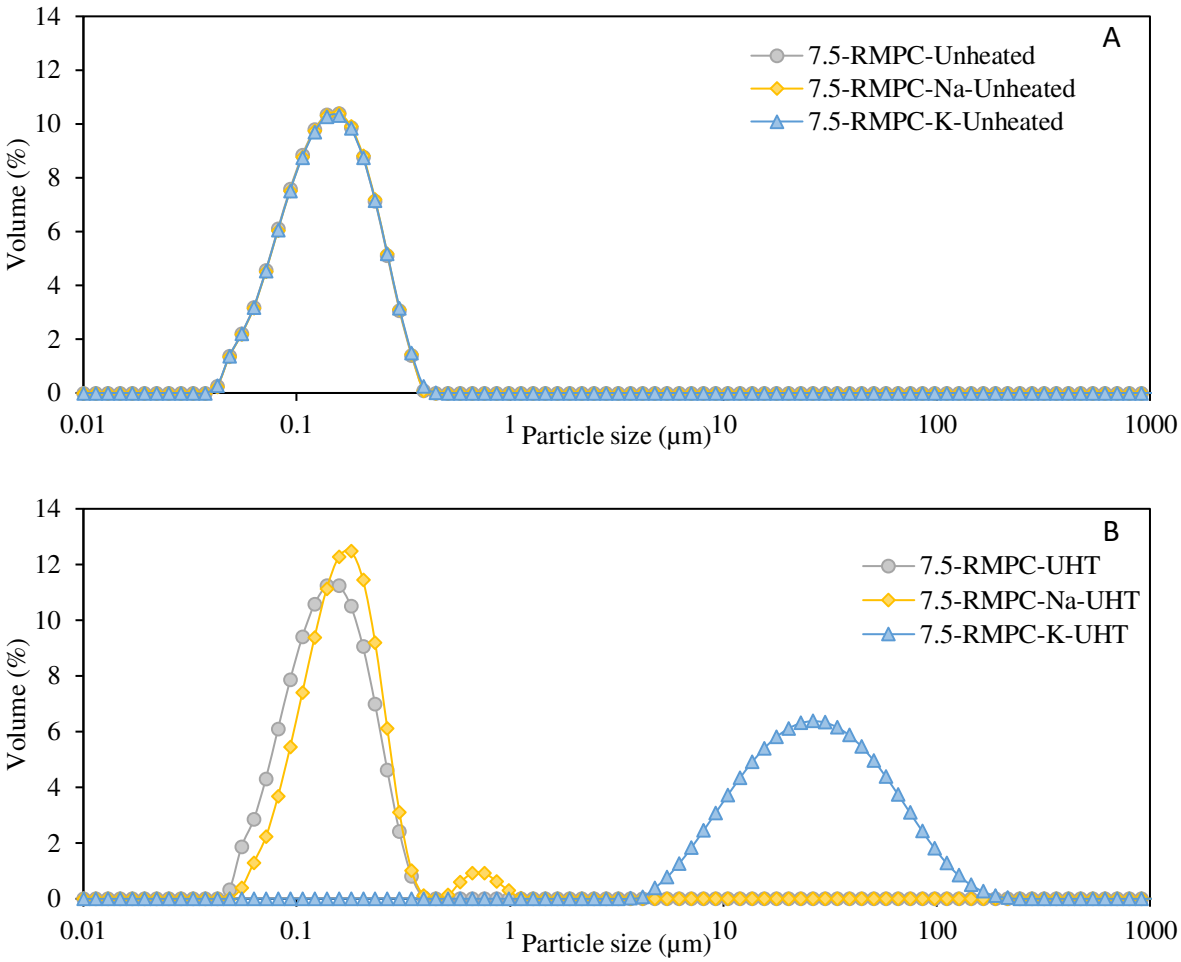

Figure 4.5: Particle size distribution of (A) unheated and (B) UHT processed milk protein dispersions. Average data of four measurements.

Table 4.3: Comparison of volume weighted mean diameter, surface weighted mean diameter and particle size distribution of milk protein dispersions.

Sample	D(4,3) (µm)	D(3,2) (µm)	D(0.1) (µm)	D(0.5) (µm)	D(0.9) (µm)
7.5-RMPC-Unheated	0.14±0.00	0.11±0.00	0.07±0.00	0.13±0.00	0.23±0.00
7.5-RMPC-UHT	0.14±0.00^{b}	0.12±0.00^{b}	0.07±0.00^{b}	0.13±0.00^{b}	0.22±0.00^{b}
7.5-RMPC-Na-UHT	0.18±0.00^{b}	0.14±0.00^{b}	0.09±0.00^{b}	0.15±0.00^{b}	0.25±0.00^{b}
7.5-RMPC-K-UHT	32.61±1.88^{a}	19.10±1.05^{a}	9.29±0.48^{a}	24.87±1.70^{a}	66.54±3.87^{a}

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different (P<0.05).

4.3.3.2 Apparent viscosity

The apparent viscosity analysis showed similar trends with PSD data. There was a slight decrease in viscosity of unheated 7.5-RMPC after salt addition. This can be related to the reduction in protein-protein hydrophobic interactions and disulphide interactions increase in solubility of milk proteins caused by salt addition (Mao et al., 2012). There was a significant change in viscosity after UHT processing of all samples (Fig 4.6). Notably, the addition of KCl to 7.5-RMPC caused a significant increase in viscosity after UHT treatment, which corresponds to the formation of larger particles as seen in PSD analyses and severe fouling results shown during its UHT processing. There was also a slight but significant increase in viscosity of 7.5-RMPC-Na after UHT processing, however there was no significant difference observed between viscosities of UHT processed 7.5-RMPC-Na and 7.5-RMPC. 7.5-RMPC showed a slight but significant decrease in viscosity after UHT treatment.

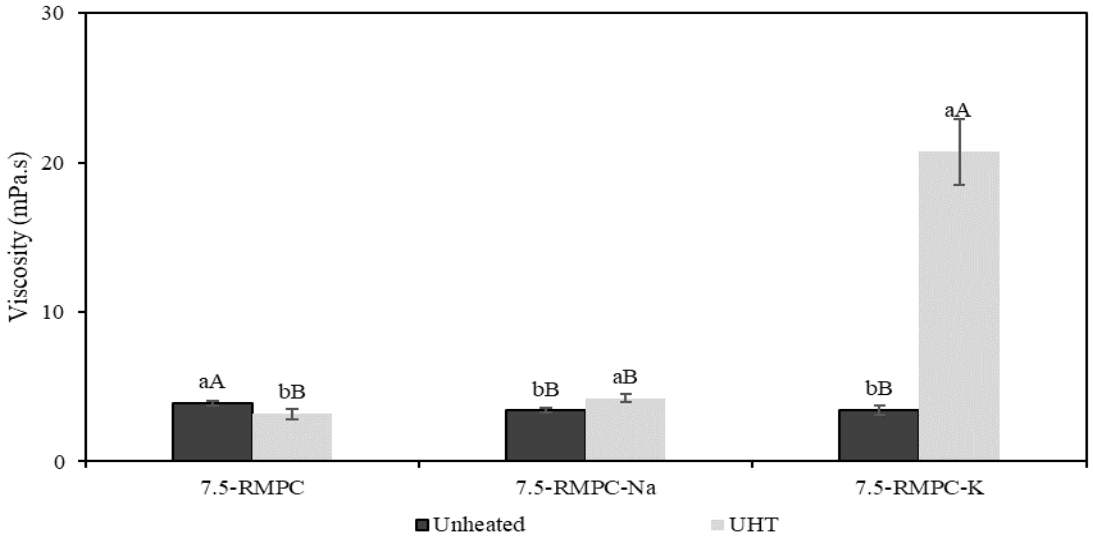

Figure 4.6: Apparent viscosity of milk protein dispersions at 300 s^{-1} shear rate, unheated vs UHT processed. Error bars represent standard deviation, n= 4. Means with different upper case letters show significant change in viscosity within the group of same sample type only (i.e. either heated or unheated), whereas different lower case letters shows significant difference among the group of same sample type (i.e. either heated or UHT processed), (P <0 .05).

4.3.3.3 Protein profile analysis

The RP-HPLC analysis was performed in order to study the effect of the addition of sodium and potassium on amounts of non-sedimentable proteins in unheated and UHT processed samples (Fig 4.7). Addition of sodium and potassium alone did not cause a significant difference in sedimentable

proteins (total as well as individual proteins) before UHT treatment (unheated samples). There was a significant decrease in non-sedimentable proteins after UHT treatment of all samples; however, it was more severe in mineral salts added samples (Fig 4.7A). In the case of NaCl and KCl added RMPC samples the total non-sedimentable protein values were significantly lower than RMPC after UHT treatment. RP-HPLC data also showed that most of the α-lactalbumin (α-la) was aggregated and β-lactoglobulin (β-lg) was completely aggregated in all the samples after UHT processing, more severely in salt-added samples. Analysis of individual casein content showed that the quantity of non-sedimentable proteins in unheated or UHT processed samples did not increase after addition of salts, this indicates that addition of NaCl and KCl did not cause any notable casein micelle dissociation. In case of UHT processed 7.5-RMPC-Na and 7.5-RMPC-K, amounts of non-sedimentable αs_1-casein, β-casein and κ-casein were significantly lower as compared to 7.5-RMPC The reduction in non-sedimentable caseins after UHT treatment could be mainly due to the increased heat induced aggregation and precipitation of serum caseins caused by salt addition (Zittle & Jasewicz, 1962).

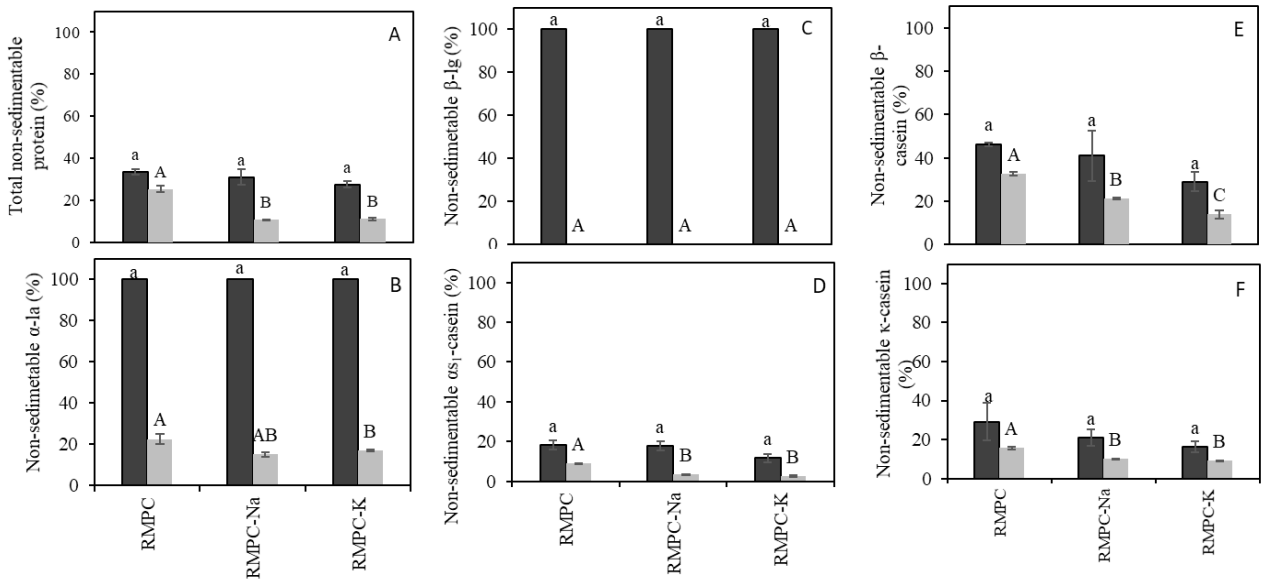

Figure 4.7: Effect of UHT processing of milk protein dispersions on non-sedimentable milk proteins (data shown as percentage of non-sedimentable protein of total individual milk protein present in the sample). (A) Total non-sedimentable protein, (B) α-la, (C) β-lg, (D) αs1-casein, (E) β-casein and (F) κ-casein . Means for proteins with different superscripts within the group of same sample type only (i.e. either heated or unheated) are significantly different (P < 0.05). Unheated = non-UHT processed sample, UHT = corresponding UHT processed sample.

The increase in ionic strength in RMPC induced by NaCl and KCl could result in a reduction in the ion activity coefficeint of calcium phosphate, increasing its solubility (Huppertz & Fox, 2006), which can destabilise casein micelle structure and increase serum casein levels. However it has been reported that increase in ionic calcium after NaCl addition was due to realease of calcium bound to caseins rather than solubilisation of calcium-phosphate nano clusters (Dalgleish & Parker, 1980; Parker & Dalgleish, 1981; Singh & Fox, 1987). Which in turn resulted in reduced dissociation of caseins from casein micelles during UHT processing as shown by RP-HPLC results. Similar results were reported by Le Ray et al. (1998). They reported a reduction in αs_1-casein and β-casein content in the supernatants of micellar casein dispersions after addition of NaCl. Singh and Fox (1987) also reported that sodium ions can shield the negatively charged groups (seryl phosphate and carboxyl groups) on casein micelles and reduce the release of κ-casein or κ-casein-whey protein complexes during heat treatment.

The heat induced aggregation of caseins and whey proteins can form fouling layers on heat exchanging surfaces. Although the reduction in amount of non-sedimentable proteins in 7.5-RMPC-Na and 7.5-RMPC-K was almost similar, much more severe fouling was observed in case of KCl added sample compared to NaCl added sample, which can be attributed to the formation of larger protein aggregates during its UHT processing (Table 4.3). The larger protein aggregates can cause rapid fouling and pressure fluctuations leading to shorter UHT run times (Skudder et al., 1981).

UHT stability and physicochemical analysis of mineral salts added 7.5-RMPC showed that increasing the concentration of potassium considerably reduced the heat stability of RMPC, whereas an increase in sodium content did not appear to have any adverse effect on the UHT stability of RMPC. Which was due to the fact that a relatively higher amount of KCl was required to raise the potassium content of 7.5-RMPC to the level of 7.5-RSMP. The protein to mineral ratio could be an important factor governing the heat stability of these two reconstituted milk powders. The concentration of some of the major minerals such as calcium, magnesium and potassium was low in RMPC as compared to RSMP when reconstituted at similar protein content, which allowed RMPC to be successfully UHT processed at very high protein concentrations. The increase in mineral concentration by addition of SMUF in 7.5-RMPC resulted in an imbalance of this equilibrium diminishing its heat stability. The ion equilibrium of cationic minerals (calcium, magnesium, potassium, sodium etc.) and other anionic minerals (phosphate, citrate, chloride etc.) in the diffusible fraction of RMPC remain in such an equilibrium that promote high heat stability of milk proteins.

4.4 Conclusions

The results from this work showed that compared to SMP lower amounts of minerals, such as calcium, magnesium and potassium in MPC were the major reason behind its high UHT stability. Increasing the calcium, magnesium and potassium concentrations to levels similar in RSMP reduced heat stability of RMPC at 7.5% protein, while the addition of sodium had no significant effect on UHT stability of RMPC. It can be concluded that the membrane filtration process used during MPC manufacturing reduces the protein to mineral ratio, which resulted in high heat stability of milk protein dispersions prepared from it. When the mineral composition was restored to the same protein to mineral ratio as found in SMP, the UHT stability of RMPC becomes similar to that of RSMP. These results can also be useful for the formulation of mineral fortified high protein beverages using RMPC.

CHAPTER 5: ULTRA HIGH TEMPERATURE (UHT) STABILITY OF CASEIN-WHEY PROTEIN MIXTURES AT HIGH PROTEIN CONTENT: HEAT INDUCED PROTEIN INTERACTIONS.

The following publication has been incorporated as Chapter 5:

Singh, J., Prakash, S., Bhandari, B., & Bansal, N. (2019). Ultra high temperature (UHT) stability of casein-whey protein mixtures at high protein content: Heat induced protein interactions. Food Research International, 116, 103-113.

Contributor	**Statement of contribution**
Jaspal Singh (Candidate)	Concept and design (85%) Analysis and interpretation (70%) Drafting and production (55%)
Sangeeta Prakash	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (10%)
Bhesh Bhandari	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (10%)
Nidhi Bansal	Concept and design (5%) Analysis and interpretation (20%) Drafting and production (25%)

Abstract: Although whey proteins have high biological value, their application to UHT processed high protein neutral beverages is limited due to their low heat stability. This research aimed to exploit the chaperone activity of caseins to improve UHT stability of whey proteins. UHT stability of reconstituted milk protein concentrate (RMPC), reconstituted whey protein concentrate (RWPC) and samples with various casein to whey protein ratios (C:W) (80:20 to 40:60) was studied. A 2% protein RWPC caused severe fouling suggesting its poor UHT stability. However, 10% protein RMPC was very stable (UHT run-time >120 min). Inclusion of caseins caused stabilization of whey proteins to UHT processing and 10% protein C:W-50:50 was successfully processed for more than 120 min. Further increase in whey proteins proportion in milk protein dispersions caused a drop in run-times (below 120 min) and overall heat transfer co-efficient (OHTC), corresponding with an increase in particle size and apparent viscosity. Presence of higher amounts of casein in the serum phase of samples caused the formation of smaller protein aggregates (D(4,3) was 0.23 and 0.16 µm for supernatants of C:W-40:60 and RMPC, respectively) after heating. These results can help to increase the whey protein content of neutral pH, UHT processed high protein ready to drink beverages.

5.1 Introduction

Bovine milk contains two main types of proteins, caseins and whey proteins (Madureira, Pereira, Gomes, Pintado, & Malcata, 2007). Whey proteins have high biological value, contain higher amounts of essential amino acids, digest rapidly in the human body and are considered superior in stimulating muscle protein synthesis in young and elderly people as compared to caseins (Burd et al., 2012; Smithers, 2008). Whey proteins consist of different fractions including β-lactoglobulin (β-lg), α-lactalbumin (α-la), immunoglobulins (Ig), lactoferrin (Lf), lactoperoxidase (Lp) and bovine serum albumin (BSA) (Smithers, 2008). Whey proteins have various physiological functions for the human body. They are a source of essential and branched chain amino acids, such as leucine. Branched chain amino acids are considered to be helpful in body fat loss and decreasing triglycerides levels in plasma (Ha & Zemel, 2003). Lf has antioxidant and antimicrobial properties along with Lp (Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002). Ig have immunomodulation benefits (Keri Marshall, 2004). Whereas caseins are a rich source of nutritionally valuable minerals such as calcium and phosphorous. Caseins are also slow digesting proteins providing amino acids to the body in a steady manner for a long duration. The overall gastric emptying time for caseins is longer than whey proteins which provides longer satiating influence on the consumer (Hall, Millward, Long, & Morgan, 2007; Tipton et al., 2004). Many bioactive peptides are released during digestion of caseins and whey proteins in the human body; these peptides have opioid, antihypertensive, immunomodulation, antimicrobial and antithrombotic functions (Chabance et al., 1998; Saito, 2008).

The use of milk protein ingredients such as whey protein concentrate (WPC), whey protein isolate (WPI) and milk protein concentrate (MPC) in high protein ready to drink beverages is becoming increasingly common due to excellent protein quality and positive health effects of milk proteins (Nagpal et al., 2011). In general, there are two types of high protein beverages, low pH acidic beverages and neutral pH (pH ~ 6.8) UHT processed or retort sterilized beverages (Beecher et al., 2008a). Milk proteins, especially whey proteins are heat labile and undergo heat-induced denaturation and aggregation during thermal processing of milk and milk protein based products (Fickak et al., 2011). Due to their low heat stability, the use of whey protein ingredients is limited to low pH acidic beverages that require lower processing temperatures than typical sterilization temperatures used for UHT and retort sterilization (Lee & Vickers, 2008). MPCs are mostly used in neutral pH beverages due to their high heat stability and ability to withstand UHT temperatures, whereas whey proteins undergo rapid aggregation and gelation during UHT processing. This restricts the use of a high percentage of whey proteins in neutral pH protein beverages (Agarwal et al., 2015; Somerscales, 1990). An increase in whey protein concentration in a beverage mix can cause heat stability problems due to the increased rate of whey protein denaturation (Law & Leaver, 1997). Milk protein instability

to thermal processing can lead to deposit formation on heat exchanging surfaces leading to shorter run times in UHT plants which requires frequent clean ups. Fouling of heat exchanging surfaces by dairy fluids is a major problem in the food industry. Whey proteins are the main contributor to fouling during UHT processing of dairy products (Changani et al., 1997).

Researchers have demonstrated the protective effect of caseins to prevent thermal aggregation of whey proteins (Guyomarc'h et al., 2009; Liyanaarachchi et al., 2015). This behaviour of caseins was assigned to their molecular chaperone like activity to protect whey proteins structure from heat induced stresses. Chaperon activity of caseins is found to be similar to small heat shock proteins (sHsp), which is attributed to their unfolded and flexible conformation. Caseins can assist in regulating the aggregation of whey proteins to form smaller soluble aggregates (Holt et al., 2013; Kehoe & Foegeding, 2011). However, most of these studies are performed on model casein systems showing chaperon activity of individual caseins (Yong & Foegeding, 2009). Typically, milk has casein to whey proteins ratio (C:W) as 80:20. MPC has caseins and whey proteins in a ratio similar to milk and has high UHT stability, whereas WPC and WPI contain entirely whey proteins (Eshpari, Tong, & Corredig, 2014). There is a need to study the effect of inclusion of caseins on UHT stability of whey proteins and up to what extent whey proteins can be stabilised against UHT thermal stresses and deposit formation on heat exchanging surfaces. Additionally, blending casein and whey protein ingredients together to achieve various ratios of casein to whey proteins in a milk protein based beverage can help to target different consumer groups such as sports, elderly and medical nutrition.

The present work focuses on utilising small amounts of casein to prevent whey protein aggregation and improve UHT stability of high whey protein beverages. The physico-chemical properties of casein-whey protein dispersions will also be studied. This will help in extending our knowledge about chaperon activity of caseins in practical applications in the food industry.

5.2 Materials and Methods

5.2.1 Materials

Commercially manufactured MPC and WPC were purchased from Maxum Foods Pty. Ltd, Australia and were used in the preparation of reconstituted milk protein dispersions for all experiments. Chemicals and reagents used in this study were purchased from Sigma-Aldrich Pty. Ltd., NSW, Australia unless otherwise stated. MPC and WPC contained on average 81.5% and 76.8% protein, respectively as per certificate of analysis provided by the supplier.

5.2.2 Preparation of reconstituted milk protein dispersions at different casein-whey protein ratios

A 10% (w/w) protein content milk protein dispersion was formulated using calculated amounts of MPC and WPC and reconstituting them in distilled water to achieve required C:W. Table 5.1 shows different C:W and sample codes used in this study. MPC85 and WPC80 were selected to prepare samples due to their protein content per gram total solids, which allowed the preparation of all samples at 10% (w/w) protein and almost similar total solids. Suffix Unheated and UHT were used to denote unprocessed and UHT processed samples, respectively. Reconstituted protein dispersions were prepared by reconstituting milk powders in distilled water at 50±2 ºC. The protein dispersions were kept under refrigeration overnight (~14 h at 3-4 ºC) to ensure complete hydration of all powder particles. Protein dispersions were then allowed to reach room temperature before further processing. The pH of protein dispersions were analysed and adjusted to 6.8 prior to UHT processing using 2M NaOH or 2M HCl, if required. Milk protein dispersions were filtered to remove any undissolved particles, if present.

Table 5.1: Description of reconstituted milk protein dispersions.

Sample	Ingredients	Protein content (% w/w)	Total solids (% w/w)	C:W ratio (% weight fraction)	C:W ratio (% molar fraction)
RMPC	MPC	10	12.27	80:20	66:34
C:W-50:50	MPC and WPC	10	12.55	50:50	32.7:67.3
C:W-45:55	MPC and WPC	10	12.60	45:55	28.5:71.5
C:W-40:60	MPC and WPC	10	12.64	40:60	24.5:75.5

5.2.3 Mineral analysis of milk protein dispersions and their supernatants

Mineral composition of samples was analysed using Inductively Coupled Plasma-Optical Emission Spectrometric (ICP-OES) analysis as described by Martinie and Schilt (1976).

5.2.4 Ionic Ca activity in reconstituted milk protein dispersions

Ca-ion activity in milk protein dispersions was measured using LAQUAtwin calcium ion meter (Horiba Instruments, Japan). The calcium ion meter was calibrated using 3.74 mM (150 ppm) and 49.90 mM (2000 ppm) Ca-ion activity standard solution before each experiment, according to manufacturer instructions. All measurements were performed in duplicate at room temperature.

5.2.5 UHT processing of reconstituted milk protein dispersions and fouling measurements

Reconstituted samples were processed using a bench-top UHT plant. The description of the UHT plant and details about conducting a UHT run and a flow diagram showing different parts of the bench-top UHT plant are presented in Chapter 3. The temperature data from the UHT plant was logged using Center 309 data logger and Microsoft Windows data logging software SE309 (Center Technology Corp., Taiwan). The preheating temperature of the product was 95 °C with 8 s holding time and sterilization temperature was 145 °C with 5 s holding time. The volumetric flow rate of the product was kept at 120 mL/min ($2X10^{-6}$ m^3/s) at the beginning of the UHT run. Duplicate experiments were conducted and the results presented are the average of the two runs.

Indicators used to end the UHT run due to deposit formation were as described in Chapter 3. Changes in overall heat transfer coefficient (OHTC) were used to monitor fouling. The plot of OHTC versus run time of UHT plant from the start to the end of the run was used to monitor the development of fouling during the UHT run.

5.2.6 Particle size distribution

Particle size distribution (PSD) of unheated and UHT processed protein dispersions was measured by dynamic light scattering (DLS) using a Malvern Mastersizer 2000MU-A (Malvern Instruments Ltd, Malvern, United Kingdom) as described by Dumpler and Kulozik (2015). The refractive index of protein was set at 1.41 and for dispersant (distilled water) was 1.33. Particle absorption index was kept as 0.001. Stirrer speed was set at 2000 rpm and laser obscuration was maintained between 10 and 11 during measurement. All measurements were performed at room temperature.

5.2.7 Optical microscopy

Microscopy images of unheated and UHT processed samples were captured to complement particle size distribution data. A small amount of sample placed on a microscopy slide was observed at a magnification of 20X under a CX-40 Olympus optical microscope (Olympus, Australia) and an Olympus digital camera (Olympus, Australia) was used to capture images.

5.2.8 Rheological analysis

The rheological data of unheated and UHT processed protein dispersions was collected using an AR-G2 Rheometer (TA Instruments Ltd., USA) using a 60 mm parallel plate geometry, The interplate gap was set at 300 µm during measurements and sample temperature was kept at 20 ºC on a Peltier plate. Measurements were performed after samples were allowed temperature equilibration for 1 min. Steady shear rate sweep test was performed on samples at shear rates from 10 to 300 s^{-1}. Power Law

model was used to calculate flow behaviour index (n) as shown in the equation below (Ibarz, Giner, Pagan, Gimeno, & Garza, 1995b):

$$\sigma = K\gamma^n \qquad \text{eq. (5.1)}$$

Where σ is the shear rate (Pa.s), K is the consistency co-efficient (Pa.s^n), γ is the shear rate (s^{-1}), and n is the flow behaviour index.

5.2.9 Preparation of ultra-centrifuged supernatants of milk protein dispersions

Unheated and UHT processed samples were ultra-centrifuged (Avanti JXN-30, Beckman Coulter, Australia Pty. Ltd., NWS, Australia) at 100,000*g* for 1 h at 20 ºC. The supernatant was removed carefully and used for further analysis.

5.2.10 Protein profile analysis

The supernatants of unheated and UHT processed samples were analysed for non-sedimentable proteins using the Kjeldahl method (AOAC, 2005).Quantification of individual non-sedimentable proteins of interest in supernatants was done using Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) (RP-HPLC-UV, Agilent 1100, Agilent Technologies Australia, Victoria, Australia) using the method adopted from Wijayanti et al. (2013).

5.2.11 Heat coagulation time measurements

Heat coagulation time (HCT) of unheated milk protein dispersions and their corresponding supernatants was measured using the method described by Davies and White (1966) with some modifications. The temperature similar to UHT sterilization (145 ºC) was used for HCT analysis. Glass vials (22.6 x 75.5 mm) containing 2 mL of sample were placed on a rocker and immersed in a temperature controlled oil bath for heating. The rocker speed was kept at ~8 revolutions per min. HCT was reported as time elapsed between putting the samples in the oil bath and appearance of first visible signs of coagulation. All experiments were performed in duplicate.

5.2.12 Statistical analysis

The data was analysed using Microsoft Excel, SigmaPlot and Minitab 16 software package. Significant differences between average values of replicate measurements at each data point was analysed by analysis of variance (ANOVA) using Tukey's HSD post hoc test at 95% confidence level.

5.3 Results and discussion

5.3.1 UHT processing of reconstituted milk protein dispersions

An initial trial to assess UHT processability of whey protein dispersion was performed on a 2% (w/w) protein sample prepared using WPC (2-RWPC). This sample caused severe fouling and complete blockage of processing line of the UHT plant (results not shown) suggesting its extremely poor UHT stability. Since RMPC showed a very high UHT stability (Fig 5.1 and 5.2), WPC was mixed with MPC and percentage of whey proteins was successively increased in the samples and UHT experiments were performed (Table 5.1).

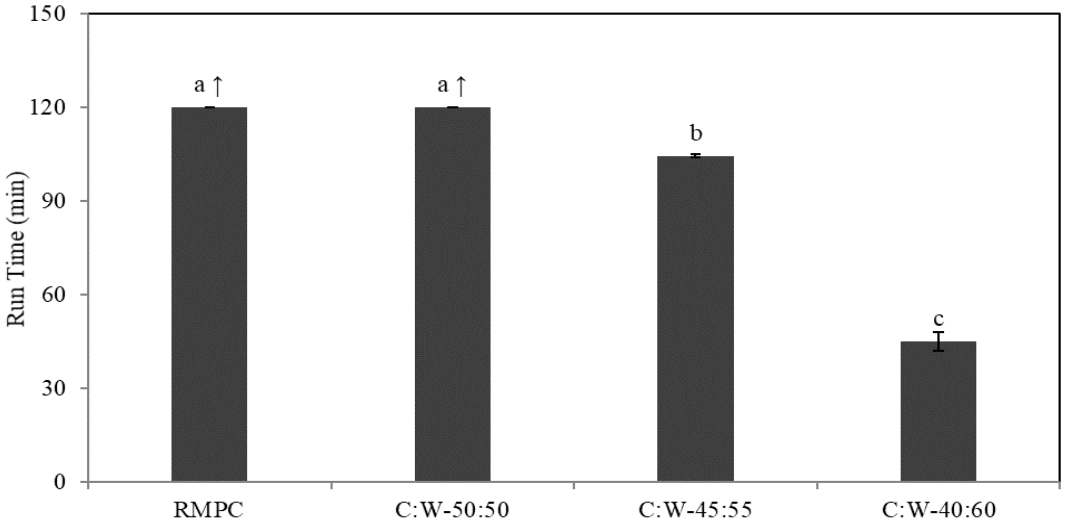

Figure 5.1: The average UHT run times on a bench top UHT tubular heat exchanger during processing of milk protein dispersions. Error bars represent standard deviation, n=2. Means with different letters are significantly different (P<0.05). Samples with ↑ did not foul in 120 min.

Assessment of UHT stability of protein dispersions was started with 10% protein RMPC, which had the native casein to whey protein ratio of 80:20. This sample showed very high UHT stability and run-time exceeded 120 min without any signs of fouling. The average run-time of the bench-top UHT plant during the processing of milk protein dispersions (10% protein) prepared with varying casein to whey protein ratios is shown in Fig 5.1. A sample prepared by increasing whey protein content to 5% (w/w), C:W-50:50, was also stable for a long period of time and UHT run-time was more than 120 min. Further increase in whey proteins in the samples decreased the run-time below the 120 min mark. The C:W-45:55 ran for 104.5 min, whereas run-time for C:W-40:60 was just 45 min. Due to excessive fouling and processing difficulties caused by sample C:W-40:60, it was decided not to increase the whey protein content in the samples any further.

The effect of different casein to whey ratios on variation in OHTC with run-time during UHT processing of samples is shown in Fig 5.2. It can be clearly observed that all samples showed similar values of OHTC at the start of UHT processing. However, for RMPC, OHTC was higher than all other samples during the UHT run and it remained almost stable until the end of the run. When compared to this sample, on an average OHTC tended to decrease with an increase in whey protein content in the samples. An initial decrease in OHTC was observed in all the samples except in RMPC (C:W-80:20). This initial decrease in OHTC became more severe with an increase in whey protein content in the samples. After an initial drop, OHTC remained almost constant before fouling started causing excessive pressure drops and temperature fluctuations in case of C:W-45:55 and C:W-40:60. These two samples showed a fouling induction period of 91 min and 32 min, respectively. The C:W-50:50 sample showed no substantial decrease in OHTC after the initial drop and lasted the entire 120 min UHT run without any severe pressure drops and temperature fluctuations.

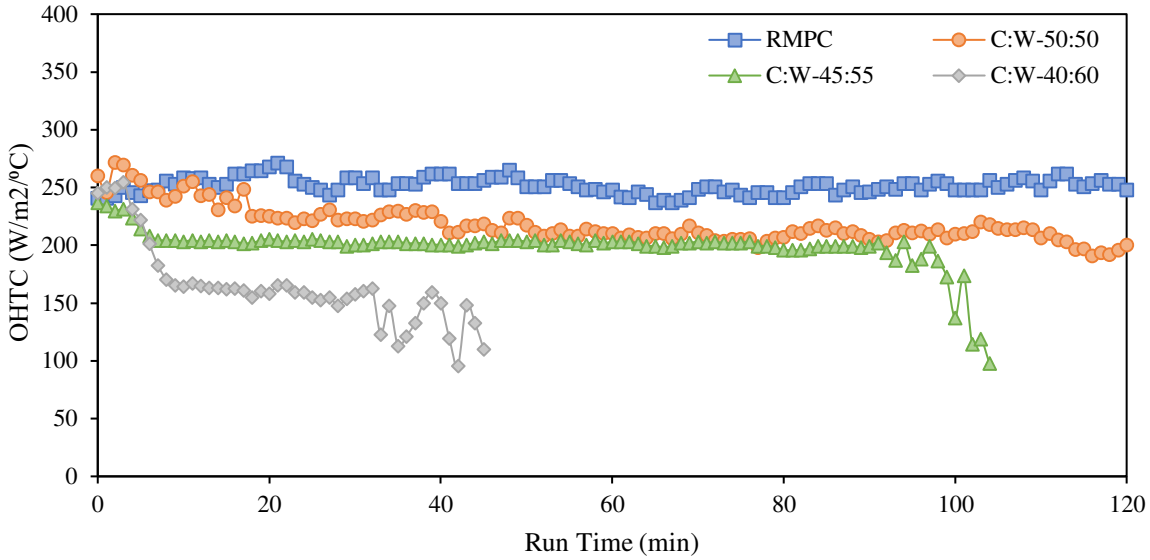

Figure 5.2: Variation in OHTC with run time for milk protein dispersions. Average data of duplicate runs is presented here.

These results showed the protective effect of chaperon activity of caseins against thermal instability of whey proteins (Liyanaarachchi et al., 2015), which allowed the inclusion of the high amount of whey proteins (=5% w/w) in UHT processed high protein dispersions possibly by controlling their extent of aggregation and aggregate sizes. However, the low thermal stability of whey proteins also played its role and increasing the whey protein concentration in the sample beyond 5% (w/w) caused the onset of rapid fouling and short UHT run-times. Excessive fouling with an increase in whey protein concentration can be related to increase in the rate of aggregation of thermally denatured β-lg molecules when they are present in the system at high concentrations (Hoffmann et al., 1997). A higher concentration of β-lg can lead to frequent interactions between protein molecules, which may

help to form larger aggregates (Le Bon et al., 1999). Larger higher molecular weight molecules can cause the formation of so called Type-A voluminous deposits (Tissier, 1984).

The initial drop in OHTC showed by all the samples except RMPC could be due to the formation of an excessive initial deposit layer, possibly caused by direct attachment of thermally activated whey proteins along with protein aggregates to the heating surfaces reducing heat transfer rate (Belmar-Beiny & Fryer, 1993). Denatured protein molecules near surfaces are considered to be more susceptible to form deposits by attaching to stainless steel surfaces whereas proteins in the bulk fluid form aggregates. Additionally, a clean metal surface possesses large free surface energy gradients and proteins are very surface active and start to adsorb on metal surfaces (Lalande et al., 1984; Santos et al., 2003). The thickness of this initial deposit layer may depend upon the amount of activated whey protein molecules and aggregates near the surface, which were possibly higher in case of samples containing higher amounts of whey proteins. Hence, the extent of the drop in OHTC during first few minutes of UHT run was possibly based upon the thickness of the initial deposit layer. After this, an induction period proceeded, where a sudden drop in OHTC by initial deposit formation was over, a gradual build-up of activated whey proteins, whey protein aggregates and whey protein-casein complexes occurred (Jeurnink, Verheul, et al., 1996).

5.3.2 Particle size distribution

Particle size distribution in unheated samples was largely in submicron range (Fig 5.3A). Whereas, particle size distribution data of UHT treated samples showed shifting in the particle size distribution of samples towards larger values as the whey protein content of the samples increased (Fig 5.3B, Table 5.2). Particle size distribution of UHT treated C:W-80:20 and C:W-50:50 was very similar and showed sub-micron particle sizes; 90% particles were below 1 μm(D(0.9) 0.23 and 0.85 μm, respectively) and 50% [D(0.5)] particles in these two UHT treated samples were less than 0.14 and 0.15 μm, respectively. Whereas particles in UHT processed C:W-45:55 and C:W-40:60 were of very large size and their D(0.9) was measured as 23.09 and 110.80 μm, respectively, similarly their D(0.5) was 3.24 and 50.52 μm, respectively. UHT processed C:W-45:55 showed almost 50% of its particles shifting to micron size range as shown by two distinct peaks for sub-micron (0.38 μm) and micron size particles (26.78 μm) in Fig 5.3B. A complete shift of particles to micron range were observed in UHT treated C:W-40:60 sample (a single peak of micron size particles, Fig 5.3B), where more than 90% of the particles [D(0.9)] were measured larger than 13.91 μm, and just 10% particles falling below this size, which was significantly higher than other three UHT processed samples.

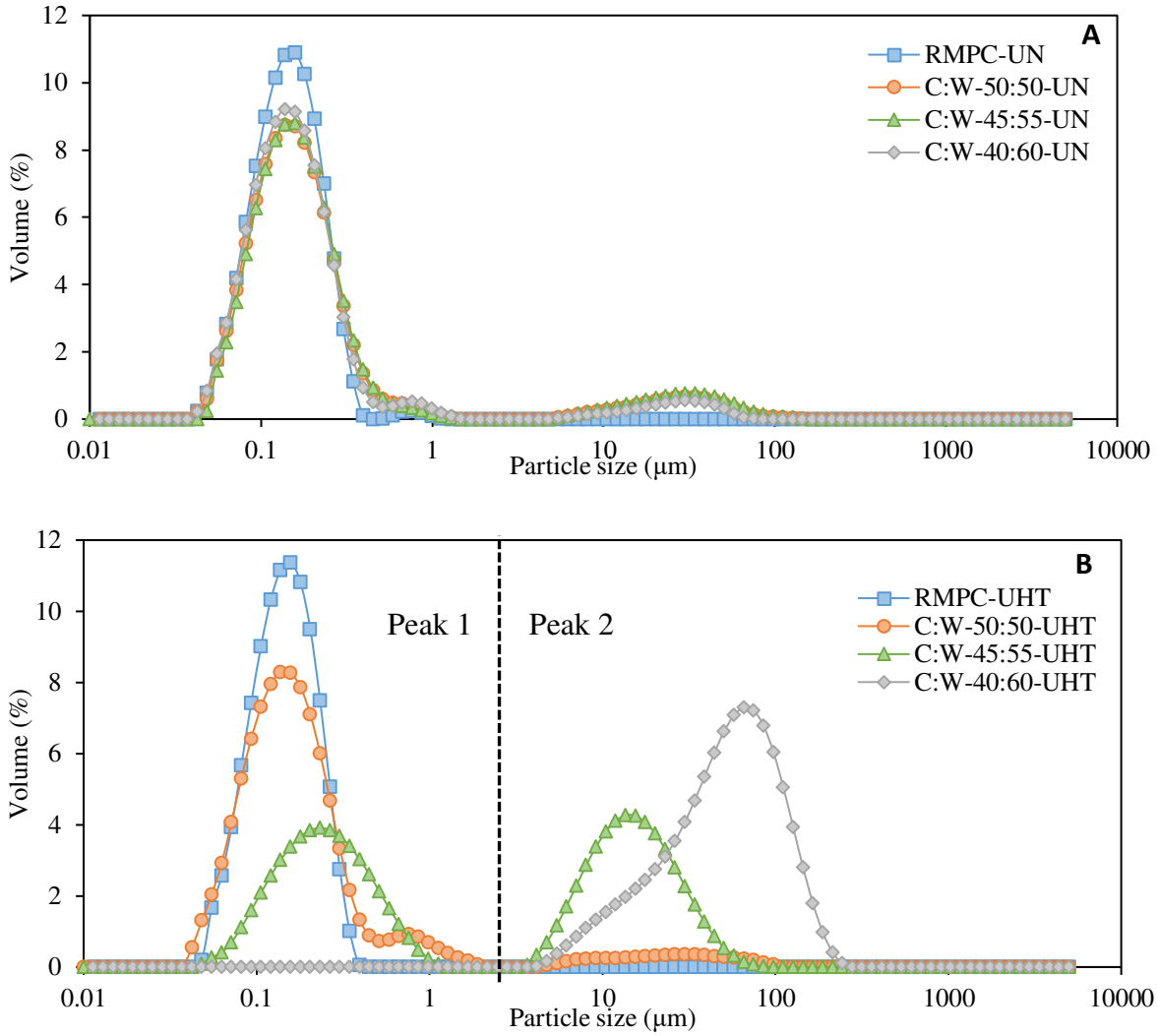

Figure 5.3: Particle size distribution of milk protein dispersions (A) before and (B) after UHT processing. Average data of four measurements.

The PSD data correlated with UHT behaviour of these samples as increasing the whey protein content increased the volume mean diameter, D(4,3), signifying the formation of larger protein aggregates causing more deposit formation and shortening the run-time. These larger particles formed are the result of reactive β-Lg unfolded form, whey protein aggregates, coagulated caseins and casein-whey protein aggregates (Anema & Li, 2003a; Blanpain-Avet et al., 2016; Dalgleish & Corredig, 2012).The surface mean diameter (D(3,2)) and D(4,3) of RMPC showed formation of compact particles. This suggests that the protein aggregates formed in this sample were likely to cause compact fouling deposits when aggregates came in the contact with surface and deposit build up will be slower as compared to other three samples (Fickak et al., 2011). In C:W-40:60 particles formed after UHT were very large. There larger particles were more likely to cause a faster build-up of deposit layer on heated surfaces and cause low UHT stability of this sample (Skudder et al., 1981). This can also be related to the difference in OHTC of samples throughout UHT processing as resistance to heat transfer is proportional to the thickness of deposit layer (Georgiadis & Macchietto, 2000; Jun & Puri, 2005).

Table 5.2: Comparison of volume weighted mean diameter, surface weighted mean diameter and particle size distribution of UHT processed milk protein dispersions.

Sample	D(4,3) (μm)	D(3,2) (μm)	D(0.1) (μm)	D(0.5) (μm)	D(0.9) (μm)	Mean diameter (μm) of particles under peaks shown in Fig 5.3B	
						Peak 1	Peak 2
RMPC	0.14±0.00^{c}	0.12±0.00^{b}	0.08±0.00^{b}	0.14±0.00^{b}	0.23±0.00^{c}	0.17±0.00^{c}	No Peak 2
C:W-50:50	1.85±1.49^{bc}	0.13±0.01^{b}	0.07±0.00^{b}	0.15±0.01^{b}	0.85±0.58^{c}	0.51±0.00^{b}	23.59±8.03^{b}
C:W-45:55	8.39±0.04^{b}	0.36±0.00^{b}	0.13±0.00^{b}	3.24±1.41^{b}	23.09±0.15^{b}	0.38±0.00^{a}	26.78±0.00^{b}
C:W-40:60	57.61±6.20^{a}	31.39±2.26^{a}	13.91±0.65^{a}	50.52±5.88^{a}	110.80±11.86^{a}	No Peak 1	61.53±7.22^{a}

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different (P<0.05).

5.3.3 Optical microscopy of milk protein dispersions

The images obtained from an optical microscope showed similar results (Fig 5.4), as observed from particle size distribution data. In general, the formation of larger particles was observed in all samples after UHT treatment. The size and number of particles gradually increased after UHT processing as the whey protein content was increased in milk protein dispersions.

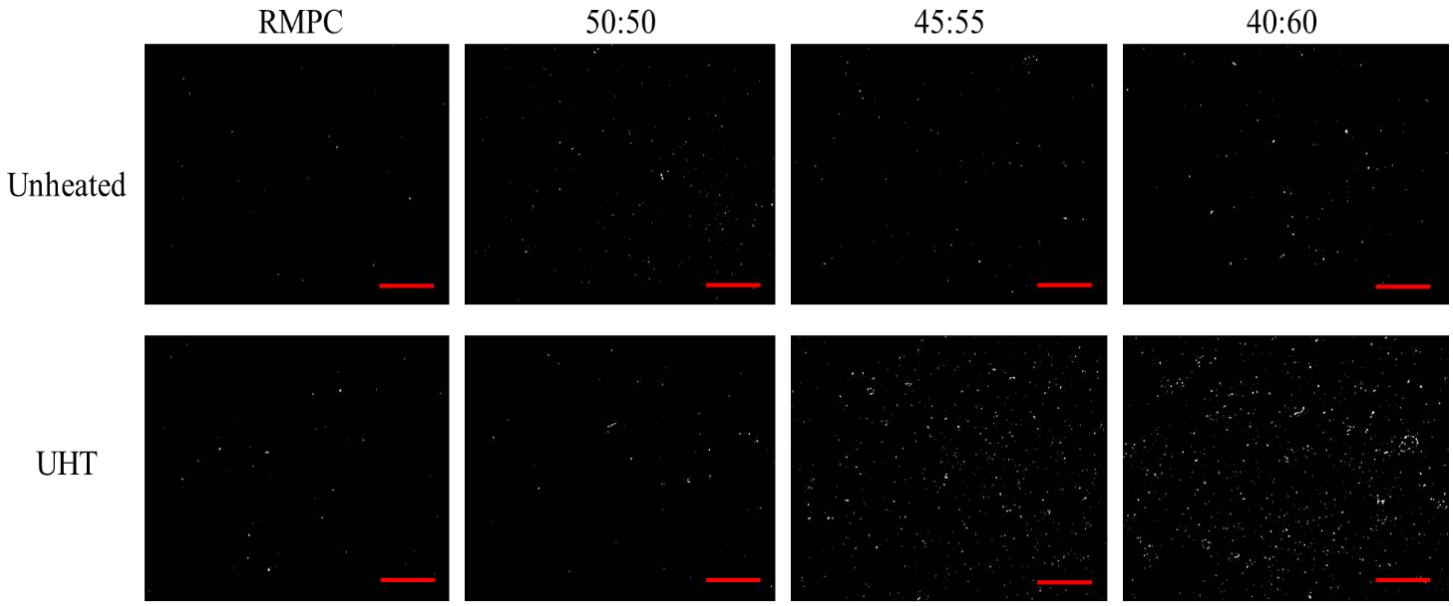

Figure 5.4: Microscopic images of unheated and UHT processed milk protein dispersion. The red scale bar represents a length of 50 µm.

5.3.4 Rheological properties of milk protein dispersions

The rheological analysis showed that there was a slight decrease in viscosity of unheated samples with an increase in whey protein content of milk protein dispersions (Fig 5.5A). Similar results were reported by Onwulata, Thomas-Gahring, and Phillips (2014) and Singh, Chandrapala, Udabage, McKinnon, and Augustin (2015). Onwulata et al. (2014) found that increasing the amount of whey proteins in protein mixes made from WPI and calcium caseinate lead to a decrease in their viscosity. Singh et al. (2015) showed that viscosity of skim milk with varied C:W increased with increase in casein content at 25 ºC which can be associated with higher viscosity of casein suspensions as compared to whey protein solutions (Boulet, Britten, & Lamarche, 1998; Hermansson, 1975). However, after UHT processing milk protein dispersions showed an increase in viscosity with increase in whey protein content (Fig 5.5A). Viscosity of C:W-50:50 was twice that of RMPC, whilst C:W-40:60 showed viscosity values 14 times higher than RMPC (apparent viscosity at a shear rate of 300 s^{-1}, which represents typical range of shear rate observed during pipe flow, mixing and stirring of liquid food products (Steffe, 1996). The whey protein gelation upon heating is greatly influenced by the presence of caseins in the system, where caseins can inhibit gel formation (Dickinson & Parkinson, 2004). This phenomenon was shown by increase in viscosity of heated milk protein dispersions when the amount of casein is decreased in them. This increase in viscosity correlated well with an increase in the particle size in samples upon UHT treatment. Flow behaviour of UHT processed milk dispersions was also analysed using the Power Law model, since they exhibited a shear thinning behaviour as seen in Fig 5.5B. It can be observed that decrease in viscosity shown by all UHT treated samples after shearing might be due to the breakdown of aggregates formed by intermolecular interactions when the shear rate is increased. The C:W-40:60 sample in particular showed significantly higher viscosity even at higher shear rates suggesting the formation of gel network that requires higher shear to break down than sample containing less whey protein content (Tang, 1993).

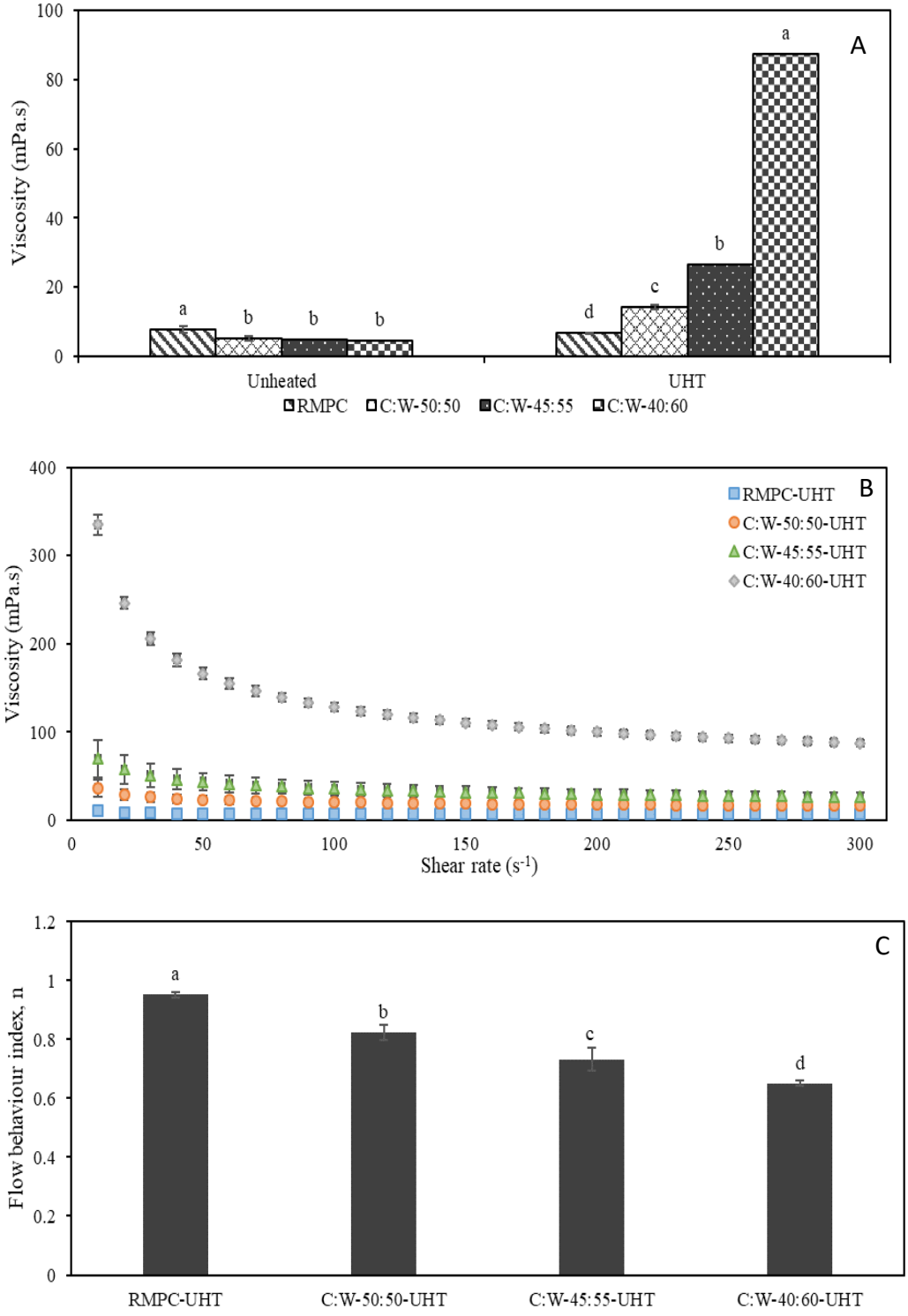

Figure 5.5: Rheological properties of milk protein dispersions (A)Apparent viscosity at 300 s^{-1} shear rate, unheated vs UHT processed (B) viscosity of UHT treated samples as a function of shear rate (C) Flow behavior index (n) of UHT processed milk protein dispersions. Error bars represent standard deviation, n=4. Means in a single column with different superscripts are significantly different (P<0.05).

The flow behaviour index (n) values gradually decreased, as shear-thinning behaviour became more pronounced with an increase in whey protein content in samples (Fig 5.5C). The C:W-40:60 sample showed the highest departure from Newtonian flow behaviour as flow behaviour index for this sample was the lowest (0.65, R^2-0.99) as compared to RMPC (0.95, R^2-0.99). This behaviour can be attributed to an increase in the formation of higher amounts of high molecular weight aggregates of denatured whey proteins during UHT processing with an increase in whey protein content in the samples.

5.3.5 Mineral composition of milk protein dispersions

MPC and WPC have different mineral composition due to differences in their processing techniques (Crowley, Megemont, et al., 2014; Morr & Ha, 1993). When these commercial milk protein powders are mixed together to formulate milk protein beverages, the resulting product has modified mineral environment and ionic strength (Yong & Foegeding, 2009).

MPC contained a higher concentration of calcium, magnesium and phosphorous than WPC, whereas sodium content was higher in WPC (Fig 5.6A). Blending MPC and WPC together resulted in a modified mineral environment in milk protein dispersions (Fig 5.6B). The mineral composition data also showed that MPC was the major contributor of calcium, magnesium and phosphorous to adjusted C:W samples. Whereas a larger proportion of sodium came from WPC and potassium was almost equally contributed by MPC and WPC. RMPC contained significantly higher amounts of calcium, sodium and phosphorous than adjusted C:W samples. High total calcium, ionic calcium and colloidal phosphate is associated with a decrease in electrostatic and steric repulsions between casein micelles, which can reduce heat stability due to increased micelle interactions (Jeurnink & De Kruif, 1995). Similarly whey protein interactions and viscosity is markedly increased with an increase in calcium levels in whey protein solutions (Tang, 1993). RMPC showed high UHT stability even when it contained higher amounts of calcium as compared to WPC added samples. However, the ionic calcium levels were significantly higher in milk protein dispersions containing WPC as compared to sample prepared from MPC only (Table 5.3). Additionally, no significant differences were observed in the ionic calcium and total calcium content amongst samples where WPC was included to vary the C:W ratio while their UHT stabilities were significantly different.

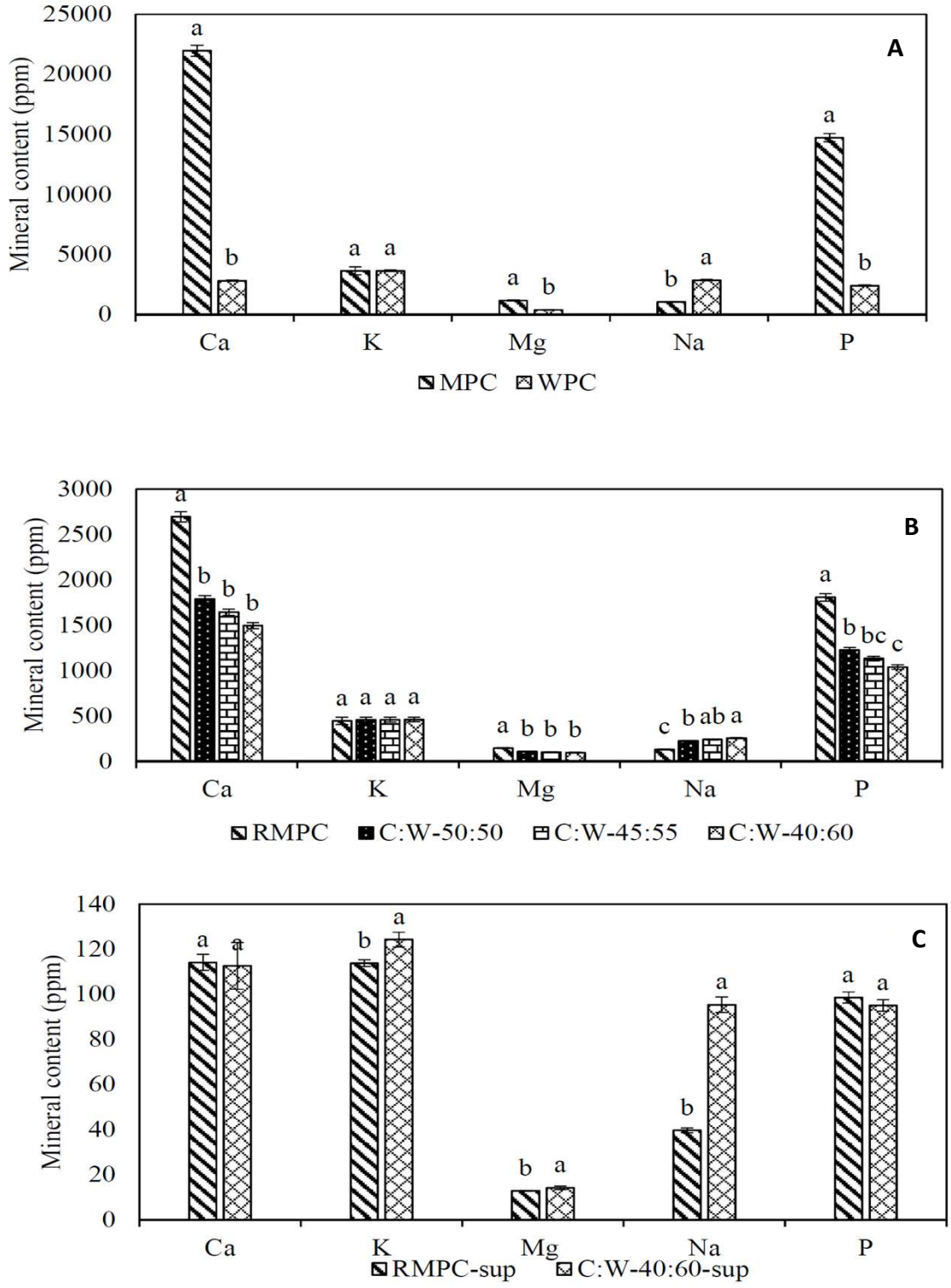

Figure 5.6: Major milk minerals (ppm) in (A) MPC and WPC powders (B) reconstituted milk protein dispersions (C) supernatants of milk protein dispersions. Error bars represent standard deviation, n=4. Bars with different superscripts are significantly different (P<0.05).

Table 5.3: Calcium ion activity in unheated milk protein dispersion samples

Sample	Calcium ion activity (mM)
RMPC	2.15 ± 0.03^{b}
C:W-50:50	2.99 ± 0.00^{a}
C:W-45:55	2.93 ± 0.11^{a}
C:W-40:60	2.93 ± 0.11^{a}

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different ($P<0.05$).

The sodium content of C:W-40:60 was almost 2.5 times higher than RMPC. This increased sodium content appeared to be responsible for an increase in calcium ion activity of milk protein dispersions with increased whey protein content. Increase in ionic strength due to high sodium content could have reduced the ionic activity coefficient of calcium phosphate, increasing its solubility and dissociation releasing more calcium in the serum (Huppertz & Fox, 2006). Supernatants of RMPC and C:W-40:60 were analysed for the mineral composition to study milk serum mineral environment (Fig 5.6C). It was found that apart from sodium, potassium and magnesium concentration of the other two major milk minerals, calcium and phosphorous, were almost similar in the two supernatants. If we compare the calcium content of milk protein dispersions and their supernatants (Fig 5.6B and C), it can be observed that serum calcium to total calcium ratio was higher in C:W-40:60 sample, which relates with high ionic calcium level observed in this sample and other samples containing WPC. It is possible that modified mineral composition could be playing a role in UHT stability of milk protein dispersions but it is very hard to compare without matching the mineral composition of these two samples. Effect of mineral composition on the heat stability of milk protein dispersions is being further investigated.

5.3.6 Protein profile analysis

Amount of non-sedimentable protein increased in unheated samples with the inclusion of higher levels of whey proteins (Fig 5.7), which was predictable. However, after UHT processing, samples with higher levels of whey proteins lost their non-sedimentable proteins in higher proportions as compared to RMPC (Fig 5.8A and B). This shows increased denaturation and aggregation of whey proteins when their levels are increased in the samples. RP-HPLC analysis showed complete

disappearance of β-lg in supernatants of all four samples after UHT treatment, whereas loss of α-la increased with an increase in whey protein content in the samples. The irreversible denaturation of α-la is favoured by the presence of higher amounts of denatured β-lg (Calvo et al., 1993; Crowley et al., 2016b). However, since the ratio of the α-la and β-lg remained same in all samples regardless of whey protein content, an increase in the concentration of β-lg may be enhancing the formation of aggregates involving α-la (Law & Leaver, 1997) leading to its almost complete denaturation in UHT processed C:W-40:60 sample.

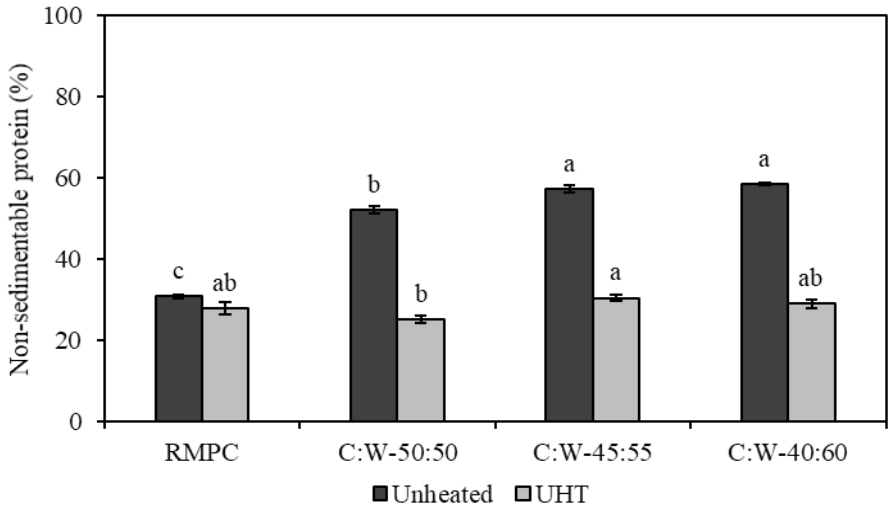

Figure 5.7: Non-sedimentable protein in milk protein dispersions (data shown as percentage of total protein of the sample). Error bars represent standard deviation, n=2. Bars with different superscripts within the group of same sample type (i.e. either heated or unheated) are significantly different (P<0.05). Unheated= non-UHT processed sample, UHT= corresponding UHT processed sample.

The non-sedimentable β-casein levels increased in unheated samples with an increase in whey protein content (Fig 5.8B). This can be related to the mineral environment of WPC used in this study. High β-casein levels could be related to high ionic calcium and release of calcium phosphate from casein micelles in the serum phase to reach equilibration (Walstra et al., 2005). This loss of calcium from casein micelles can lead to dissociation of loosely bound β-casein from casein micelles, which increased the non-sedimentable casein content in samples (Dalgleish & Law, 1988). However, less than 60% loss of β-casein (Fig 5.8C) could not cause disintegration of casein micelles and contributed to low UHT stability of samples containing WPC. The α_s-casein is largely responsible for casein micelles framework (Lin, Leong, Dewan, Bloomfield, & Morr, 1972), α_s-casein and κ-casein were not detected in the supernatant of samples containing WPC (the very small amount was present in sample RMPC, data not shown). The non-sedimentable β-casein in UHT processed samples showed

a decline similar to total non-sedimentable protein content (Fig 5.7 and 5.8) suggesting protein aggregate formation during UHT processing.

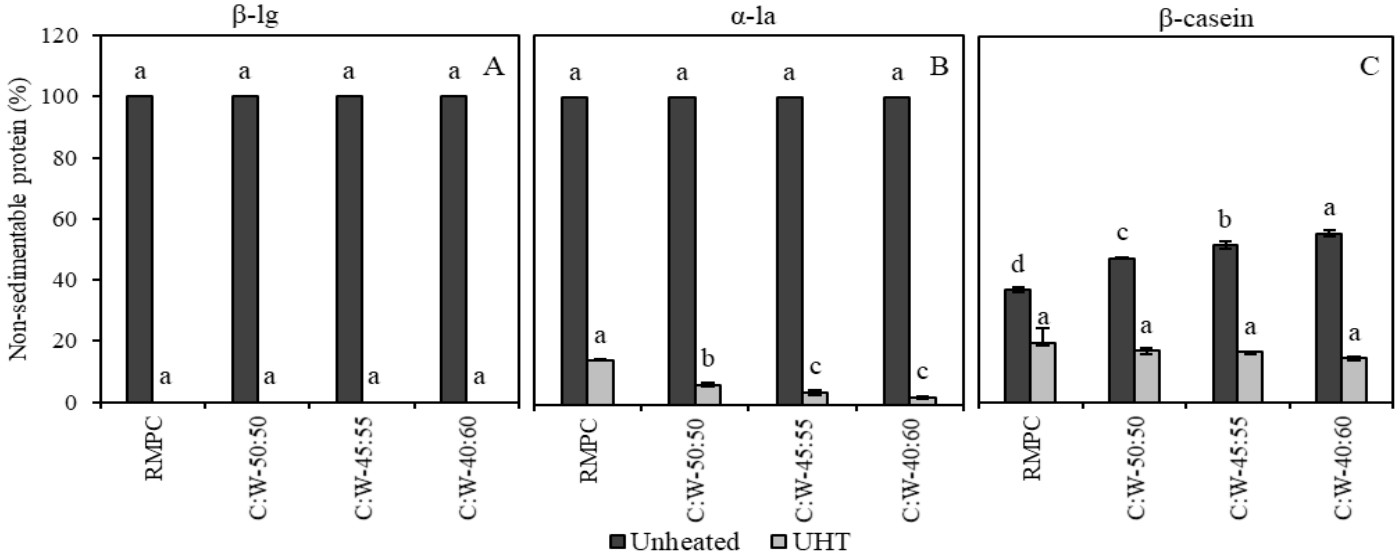

Figure 5.8: Effect of UHT processing of milk protein dispersions on non-sedimentable major milk proteins (data shown as percentage of non-sedimentable protein of total individual milk protein present in the sample). (A) β-lg, (B) α-la and (C) β-casein. Error bars represent standard deviation, n=2. Bars for individual proteins with different superscripts within the group of same sample type (i.e. either heated or unheated) are significantly different (P<0.05). Unheated= non-UHT processed sample, UHT= corresponding UHT processed sample.

Hence, it can be suggested that the higher ionic Ca content in samples with added whey proteins could have decreased casein micelle stability and led to formation of larger aggregates by enhancing aggregation of whey proteins amongst themselves and/ or with dissociated caseins, as well as increasing aggregation of destabilized casein micelles (Anema & Li, 2000; Anema & Klostermeyer, 1997; Anema & Li, 2003b; Ono et al., 1999).

5.3.7 Thermal stability of serum-phase proteins and their aggregate formation after heating

Addition of WPC to MPC resulted in milk protein dispersions with modified serum protein composition (Fig 5.7 and 5.8). The whey proteins were found in the serum phase of resulting milk protein dispersions along with dissociated caseins (Fig 5.7 and 5.8). To assess the effect of modified serum phase on the heat stability of milk protein dispersions, RMPC and C:W-40:60 and their supernatants were selected for HCT analysis (Table 5.4). Since the amount of casein (primarily β-casein) was different in the serum phase of these two samples, it would be interesting to analyse the protective effect of caseins on whey proteins during heating of supernatants. Moreover, The stability

of milk proteins at UHT temperatures can be influenced by heat induced aggregation and gelation of serum phase proteins (Singh, 2004). HCT of these two samples and their supernatants corroborated with their UHT stability. RMPC and its supernatant showed significantly higher HCT as compared to C:W-40:60 and its supernatant. However, it is important to consider that protein content of C:W-40:60-sup was almost three times higher than RMPC-sup. Therefore, C:W-40:60-sup was diluted with deionized water to achieve protein content similar to RMPC-sup. HCT of diluted sample was determined in order to look into the effect of chaperon activity of caseins on the heat stability of supernatants at similar whey protein content. Which showed an increase in HCT of C:W-40:60-sup but still significantly less than RMPC-sup. However, HCT of diluted C:W-40:60 was still significantly higher than the HCT of 2-RWPC.

Table 5.4: HCT of milk protein dispersions and supernatants.

Sample	HCT (min)
RMPC	6.65 ± 0.28^{a}
C:W-40:60	0.82 ± 0.11^{b}
RMPC-sup	13.43 ± 0.92^{a}
C:W-40:60-sup	0.76 ± 0.07^{d}
Diluted C:W-40:60-sup (Protein content similar to RMPC-sup)	9.23 ± 0.21^{c}
2-RWPC	5.91 ± 0.59^{b}

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different ($P<0.05$).

Suffix "–sup" followed by sample name represents its ultracentrifuge supernatant.

If we look into the amount of dissociated β-casein in the RMPC-sup and C:W-40:60-sup (Fig 5.8C), C:W-40:60-sup and diluted C:W-40:60-sup contained lower β-casein to whey ratio as compared to RMPC-sup (Table 5.5), whereas 2-RWPC was solely comprised of whey proteins. Low HCT of diluted C:W-40:60-sup was possibly due to the fact that relatively less dissociated casein (β-casein) was available to act as chaperon protein as compared to RMPC-sup. Therefore, it is suggested that chaperon activity of dissociated β-casein in these two supernatants appears to be the major factor affecting their heat stability.

Table 5.5: Protein distribution in supernatants of RMPC and C:W-40:60.

Sample	Whey protein (%w/w)*	Casein (dissociated β-casein) (%w/w)#
RMPC-sup	2	1.05
C:W-40:60-sup	6	1.58
Diluted C:W-40:60-sup (Protein content similar to RMPC-sup)	2	0.53

*Calculated values from Table 5.1.

#quantified using RP-HPLC.

The effect of UHT temperature on PSD of RMPC-sup, diluted C:W-40:60-sup and 2-RWPC were analysed after heating them in an oil bath at 145 ºC for 5 min (Fig 5.9). In heated diluted C:W-40:60-sup and 2-RWPC some particles in micron range were observed as compared to heated RMPC-sup, whose PSD was entirely in the sub-micron range. Larger particles were formed after heating diluted C:W-40:60-sup (protein content similar to RMPC-sup) as compared to RMPC-sup; their (D(4,3) was 0.23±0.06 and 0.16±0.00 respectively). The behaviour of milk proteins in the serum phase at UHT temperatures governed by whey protein aggregation and availability of protective dissociated caseins appeared to be an important factor in observed HCT and PSD results (Crowley et al., 2015). The UHT stability and physico-chemical data strongly suggest that the chaperon activity of caseins and protein-protein interactions appears to be the major cause behind improved UHT stability of whey proteins with the inclusion of caseins.

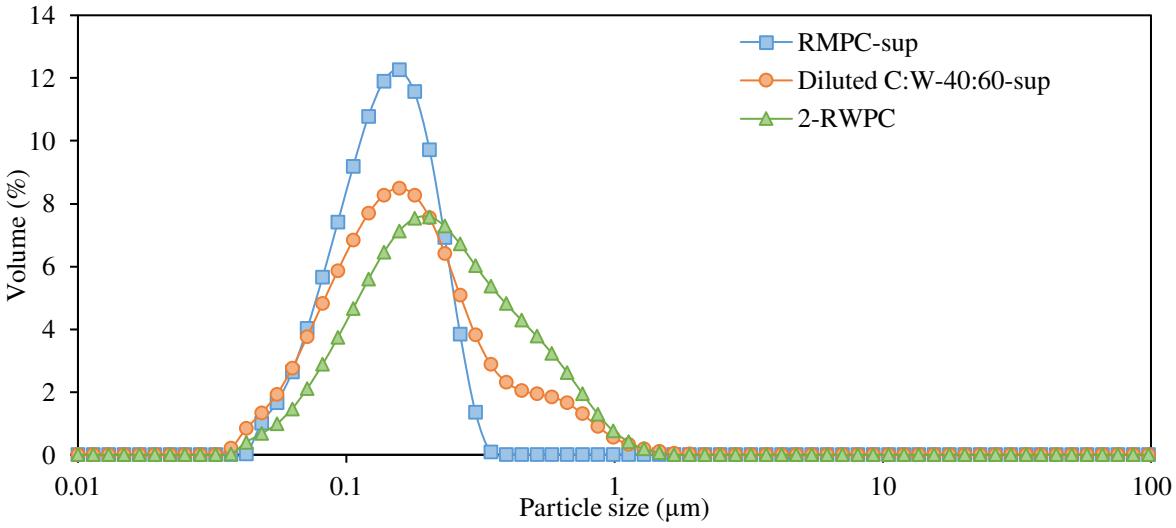

Figure 5.9: Particle size distribution of supernatants (heated at 145 ºC for 5 min) of UHT processed milk protein dispersions. Average data of four measurements.

5.4 Conclusions

High concentrations of whey proteins (=5% w/w) were successfully UHT processed by the inclusion of caseins in the milk protein dispersions. However, further increasing the amount of whey proteins in the mix was not possible due to high levels of fouling induced in samples containing 6% (w/w) whey proteins. Modified mineral composition of milk protein dispersions did not appear to significantly affect their UHT stability. Therefore, it can be concluded that the protective effect of caseins working as chaperon proteins was the major factor that controlled and suppressed whey protein aggregation. These results can help in increasing the whey protein content in commercial high protein beverage mixes.

CHAPTER 6: UHT STABILITY OF CHOCOLATE FLAVOURED HIGH PROTEIN BEVERAGES

The following submitted manuscript has been incorporated as Chapter 6:

Singh, J., Prakash, S., Bhandari, B., & Bansal, N. (Submitted in February, 2020). Ultra high temperature (UHT) stability of chocolate flavoured high protein beverages, under review in Journal of Food Science.

Contributor	**Statement of contribution**
Jaspal Singh (Candidate)	Concept and design (85%) Analysis and interpretation (70%) Drafting and production (55%)
Sangeeta Prakash	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (10%)
Bhesh Bhandari	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (10%)
Nidhi Bansal	Concept and design (5%) Analysis and interpretation (20%) Drafting and production (25%)

Abstract: Effect of two milk protein formulation bases and κ-carrageenan concentration (0, 0.01, 0.03 and 0.05%) on UHT fouling behaviour of chocolate flavoured high protein beverages was studied. The two beverage bases prepared were: i) reconstituted milk protein concentrate (RMPC-Choco) and ii) RMPC and reconstituted whey protein concentrate (C:W-Choco). Physical and sensory properties of the beverages were also investigated. The UHT run-times for samples prepared without the addition of κ-carrageenan were very short (9.5 ± 0.71 and 26.5 ± 2.12 min for RMPC-Choco-0 and C:W-Choco-0, respectively) due to settlement of cocoa powder particles in the UHT plant leading to severe fouling. It was found that addition of 0.03 and 0.01% κ-carrageenan to RMPC-Choco and C:W-Choco, respectively, made them UHT stable (UHT run-times >120 min) due to formation of a weak gel by milk protein- κ-carrageenan interactions entrapping cocoa particles. Additional gelation of higher amounts of whey proteins in C:W-Choco were linked with low levels of κ-carrageenan required for this sample to become UHT stable. This additional gelation was evident from larger average particle sizes and higher viscosity of UHT treated C:W-Choco samples when compared to RMPC-Choco samples with similar κ-carrageenan concentrations. Consequently a loss of fluidity in C:W-Choco samples with 0.03 and 0.05% κ-carrageenan was observed. A panel of untrained sensory test panellists did not found any significant difference in overall preference among three selected sample (RMPC-Chcoc with 0.03 and 0.05% κ-carrageenan and C:W-Choco with 0.01% κ-carrageenan).

6.1 Introduction

Ready to drink (RTD) high protein beverages are protein supplement drinks formulated from milk proteins ingredients and some plant proteins sources (Agarwal et al., 2015). These nutritional beverages are consumed as a convenient source of high quality milk protein to provide amino acids for muscle maintenance, energy and electrolyte replacement and for rehydration purposes (Chen & O'Mahony, 2016; Whitney et al., 2011). The nutritional profile of high protein beverages can also be specifically tailored to target different consumer groups such as a meal replacement drink to target ordinary consumers or performance enhancement drink for sportspersons (Cockburn et al., 2010). These products are formulated mainly using milk protein ingredients, flavours, carbohydrates and stabilisers to achieve desired macro and micro nutrients content and sensory attributes (Baxter et al., 2011; Lönnerdal & Hernell, 1998). High protein beverages are flavoured to increase their consumer acceptance (Bisig & Kelly, 2017). The chocolate flavour is one of the most popular flavour used in milk protein based flavoured beverages (Yanes, Durán, & Costell, 2002b).

According to Food and Drug Administration (FDA) of United States, RTD high protein drinks should contain a minimum of 10 g protein (~4.2%) and less than 18 g sugar (~7.5%) per 240 mL of drink (Etzel, 2004). Therefore some milk protein powders are highly suitable for the formulation of these beverages. Milk protein concentrate (MPC) and whey protein concentrate (WPC) contain high amounts of milk proteins (50-85%) per dry solid content and are suitable to use as primary ingredients in high protein beverages (Pandalaneni, Amamcharla, Marella, & Metzger, 2018). MPC contains caseins and whey proteins in their original proportions as found in milk, which makes MPC an excellent source of complete milk protein and native micellar casein (Augustin et al., 2011). WPC can be added to product formulations to increase the amount of whey proteins in high protein beverage due to its low amounts of non-protein components (Huffman & James Harper, 1999).

Chocolate flavoured high protein beverages are generally produced at near neutral pH (~6.7-6.8) (Beecher et al., 2008b). Ultra high temperature (UHT) processing is a product sterilisation technology commonly used to process neutral pH RTD beverages to make them shelf stable for an extended period of time (Clare et al., 2005). Heat exchanger surface fouling due to denaturation and aggregation of milk proteins is a major problem associated with UHT processing of milk protein dispersions (Deeth, 2010). This problem was addressed in previous chapters and it was found that reconstituted MPC (RMPC) up to 14% protein content can be successfully UHT processed without any notable fouling impairing its UHT processing (Chapter 3). Additionally, high protein dispersions with increased amounts of whey proteins prepared from RMPC and reconstituted WPC (RWPC) were also successfully UHT processed (casein-whey protein mixtures containing 50% caseins and 50% whey

proteins) (Chapter 5). Therefore, these two milk protein dispersions can be used as suitable base ingredients to formulate chocolate flavoured drinks. A chocolate drink contains cocoa powder as a flavouring agent and a large part of cocoa powder particles is insoluble in milk protein dispersions and some cocoa particles will settle to the bottom of the container during storage under the influence of gravity (Bixler, Johndro, & Falshaw, 2001). κ-carrageenan is a hydrocolloid which is the most commonly used stabiliser in milk protein based chocolate drinks to delay the cocoa particles sedimentation during long term storage (Hansen, 1993; Langendorff, Cuvelier, Michon, Launay, & Parker, 2000). A weak gel formation by carrageenan entraps cocoa particles and prevents them from sedimentation (Yanes et al., 2002b). The weak carrageenan gel and carrageenan-casein network formation also imparts favourable mouthfeel to low fat high protein drinks (Bixler et al., 2001).

Formulation of a chocolate drink requires the addition of various ingredients to milk protein dispersion base to facilitate processing and storage stability and additionally to provide them with acceptable sensory qualities (Bisig & Kelly, 2017). Addition of cocoa powder, sugar and hydrocolloids can significantly alter the UHT stability of a milk protein dispersion base due to change in their fouling behaviour (Prakash, Huppertz, Karvchuk, & Deeth, 2010). This change in UHT stability of chocolate drink could be due to altered rheological properties and interaction among various ingredients and milk proteins when compared to the UHT processing behaviour of milk protein dispersion base alone (Boomgaard et al., 1987).

The present work was aimed at studying the UHT fouling behaviour of chocolate flavoured high protein beverage to identify suitable amounts of κ-carrageenan to increase their UHT stability. Additionally, some physical and sensory properties of chocolate drink samples were also investigated.

6.2 Materials and Methods

6.2.1 Materials

Commercially manufactured MPC and WPC (supplied by Maxum Foods Pty. Ltd., Australia) were used as milk protein ingredients in the preparation of chocolate flavoured high protein beverage. Cocoa powder (Dutch process) and κ-carrageenan were purchased from Melbourne Ingredient Depot, Australia. Sucrose was purchased from a local supermarket. MPC and WPC contained on average 81.5% and 76.8% protein, respectively, as per certificate of analysis provided by the supplier.

6.2.2 Formulation of chocolate flavoured high protein drink

Two base milk protein dispersions containing 10% (w/w) protein were prepared from MPC only and an MPC and WPC mix by reconstituting them in distilled water (50±2°C) as described in Chapter 5.

1.5% (w/w) cocoa powder and 7% (w/w) sucrose were added to the base milk protein dispersions according to typical composition of a chocolate milk (Boomgaard et al., 1987; Prakash et al., 2010). Concentrations of κ-carrageenan were selected according to the recommended concentration range (0.01-0.05%) used in chocolate milk formulation (Bisig & Kelly, 2017). The pH of all the samples was monitored and adjusted to 6.8 before UHT processing. A complete description of the samples used in this study is presented in Table 6.1.

Table 6.1: Description of chocolate flavoured high protein drink samples.

Sample Name	Base milk protein dispersion	κ-carrageenan concentration (w/w %)	Remarks
RMPC-Choco-0	Reconstituted MPC	0	All samples contain 1.5% (w/w) cocoa powder and 7% (w/w) sucrose.
RMPC-Choco-0.01		0.01	
RMPC-Choco-0.03		0.03	
RMPC-Choco-0.05		0.05	
C:W-Choco-0	Reconstituted MPC and WPC to achieve a casein to whey protein ratio (C:W) of 50:50	0	Protein content of all samples was 10% (w/w). Total solids content of all samples was 21 ± 0.2% (w/w).
C:W-Choco-0.01		0.01	
C:W-Choco-0.03		0.03	
C:W-Choco-0.05		0.05	

6.2.3 UHT processing and fouling measurements

All samples were UHT processed using a bench-top UHT plant. A complete description and flow diagram showing different parts of the UHT plant can be found in Chapter 3. In the UHT plant, samples were preheated to 95 °C for 8s and then sterilised at 145 °C for 5s. The volumetric flow rate of the product was kept at 120 mL/min (2×10^{-6} m^3/s) at the beginning of the UHT run. Change in overall heat transfer coefficient (OHTC) was calculated by recording changes in preheating and sterilisation temperatures during a UHT run and was used to monitor fouling behaviour of samples as described in Chapter 3. All experiments were conducted in duplicate and the results presented are the average of two runs.

6.2.4 Particle size distribution

Particle size distribution (PSD) of unheated and UHT processed protein dispersions was measured by dynamic light scattering (DLS) using a Malvern Mastersizer 2000MU-A (Malvern Instruments Ltd., Malvern, United Kingdom) as described by Dumpler and Kulozik (2015). The refractive index of protein was set at 1.41 and for dispersant (distilled water) was 1.33. Particle absorption index was kept as 0.001. Stirrer speed was set at 2000 rpm and laser obscuration was maintained between 10 and 11 during measurement. All measurements were performed at room temperature (23 °C).

6.2.5 Rheological measurements

The flow behaviour of unheated and UHT processed protein dispersions was analysed using an AR-G2 Rheometer (TA Instruments Ltd., USA) equipped with a 60 mm parallel plate geometry with an interplate gap set at 300 µm during measurements. The sample temperature was kept at 20 °C using a Peltier plate. Rheological measurements were performed after allowing the sample equilibration for 1 min. Steady shear rate sweep test was performed on samples at shear rates from 10 to 300 s^{-1} (See Chapter 5 for details).

6.2.6 Visual observation of samples and sedimentation measurements

After a storage period of 48 h at room temperature (22–25 °C) the unheated and UHT processed samples were photographed for the extent of sedimentation of cocoa particles under the influence gravity. Sedimentation in UHT processed samples was quantitatively measured using a centrifuge (3000*g* for 15 min) and oven drying (120 °C for 36 h) method as described by Prakash et al. (2010). The results were presented as g of sediment/ 100g of the sample (g/100g). In order to account for precipitation of large protein aggregates with cocoa particles, the protein content of dried sediments was measure using the Kjeldahl method (AOAC, 2005).

6.2.7 Colour measurements

A Minolta Konica Chroma Meter CR-400 (Konica Minolta, INC, Japan) was used to measure the colour of samples and Hunter L, a and b values for colour parameters were recorded. The instrument was calibrated using a white tile (Y = 94.93, x = 0.3131, y = 0.3197) before sample measurements.

6.2.8 Sensory evaluation

Selected samples were tested for various sensory attributes by asking 40 untrained volunteers to rank them for either intensity of a specific attribute or for general preference. Sensory attributes evaluated were: appearance (visual appeal, colour), thickness, smoothness, creaminess, residual coating, astringency and overall preference. A detailed description about these sensory attributes can be found

in Ningtyas, Bhandari, Bansal, and Prakash (2019) and Deshpande, Chinnan, and McWatters (2005). Samples filled in small cups (30 mL) were served at room temperature in random order and evaluated by each panellist in individual booths. Panellists were instructed to rinse their mouths with water before each sample. Ethics approval for sensory evaluation involving human volunteers was obtained from The University of Queensland (Approval number 2019001010).

6.2.9 Statistical analysis

The data obtained from all experiments was analysed using Microsoft Excel, SigmaPlot and Minitab 16 software package. Significant differences between average values of replicate measurements at each data point was analysed by analysis of variance (ANOVA) using Tukey's HSD post hoc test at 95% confidence level. For sensory analysis Friedman's test was used (95% confidence level) for ANOVA. Fisher's Least Significant Difference (LSD) test was used to determine significant difference between samples for pairwise comparisons.

6.3 Results and discussion

6.3.1 UHT stability of chocolate flavoured high protein beverages

Two milk protein dispersions (RMPC and C:W) were selected to prepare chocolate beverages in the present work based on their high UHT stability as shown previously in chapter 5. RMPC contained casein to whey protein ratio similar to cow's milk (80:20) and in comparison C:W had elevated whey protein content (casein-whey protein ratio of 50:50). The UHT stability of control samples without added stabilizer (κ-carrageenan) and samples with different levels of κ-carrageenan concentration was studied by monitoring variation in OHTC with processing time during their UHT processing.

Both of the control samples (RMPC-Choco-0 and C:W-Choco-0) showed very short UHT run-times, however C:W-Choco-0 lasted significantly longer (26.5 ± 2.12 min) than RMPC-Choco-0 (9.5 ± 0.71 min) under UHT processing before fouling (Fig 6.1). Addition of 0.01% κ-carrageenan increased the UHT run-time of RMPC-Choco significantly and further increase in κ-carrageenan concentration (0.03 and 0.05%) improved its UHT stability sufficiently to render this formulation UHT stable for more than 120 min. In comparison, 0.01% of κ-carrageenan was sufficient to improve UHT stability of C:W-Choco to an extent where it can be processed for more than 120 min without any noticeable fouling. However with further increase in κ-carrageenan level (0.03 and 0.05%) in this formulation, the UHT processed samples became highly viscous and started showing gel like behaviour (rheological and particle size distribution data is presented in subsequent sections).

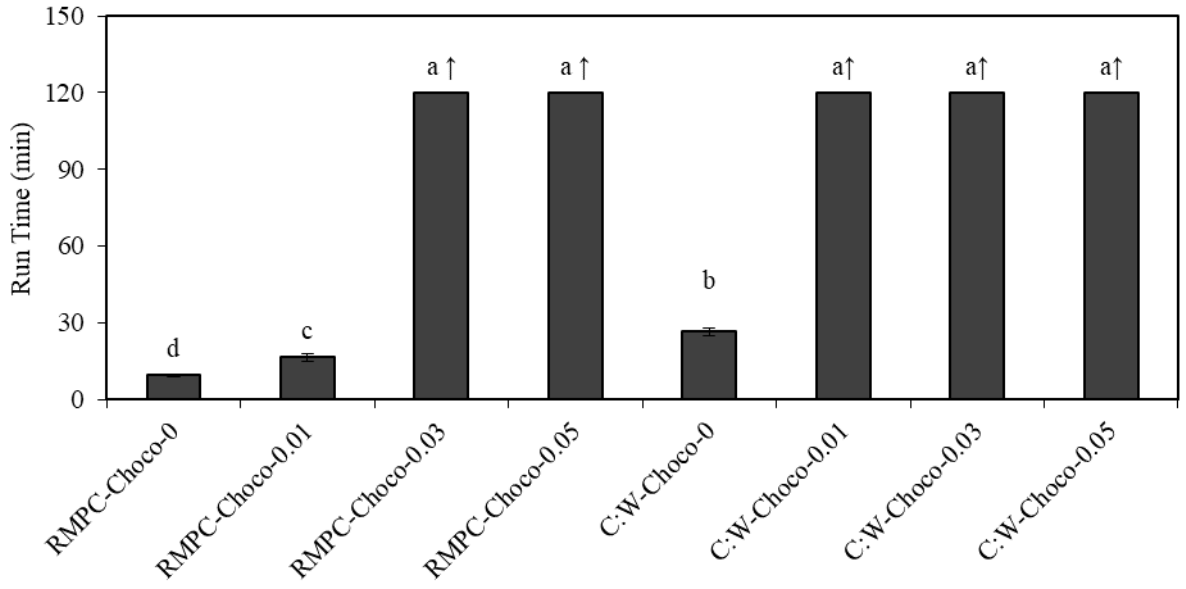

Figure 6.1: The average UHT run times on a bench top UHT tubular heat exchanger during processing of chocolate flavoured high protein beverages. Error bars represent standard deviation, n=2. Means with different letters are significantly different (P<0.05). Samples with ↑did not foul in 120 min.

Fig 6.2A and B show fouling behaviour of RMPC-Choco and C:W-Choco formulations, respectively, as represented by variation in OHTC with UHT run-time. During UHT processing of both control samples severe pressure fluctuations were observed from the starting of their UHT runs, which resulted in very short UHT runs. Addition of κ-carrageenan to both formulations (except RMPC-Chcoco-0.01) stabilized them to UHT processing and improved their UHT stability. It can be observed from Fig 6.2A that OHTC for RMPC-Choco formulations was largely unaffected by κ-carrageenan concentrations throughout their UHT run. Whereas during UHT processing of C:W-Choco formulations, OHTC of sample with 0.01% κ-carrageenan was higher than control samples, however further increase in κ-carrageenan concentrations (0.03 and 0.05%) caused a reduction in OHTC without any severe fouling that may lead to an early shut-down of UHT plant (before 120 min mark) (Fig 6.2B).

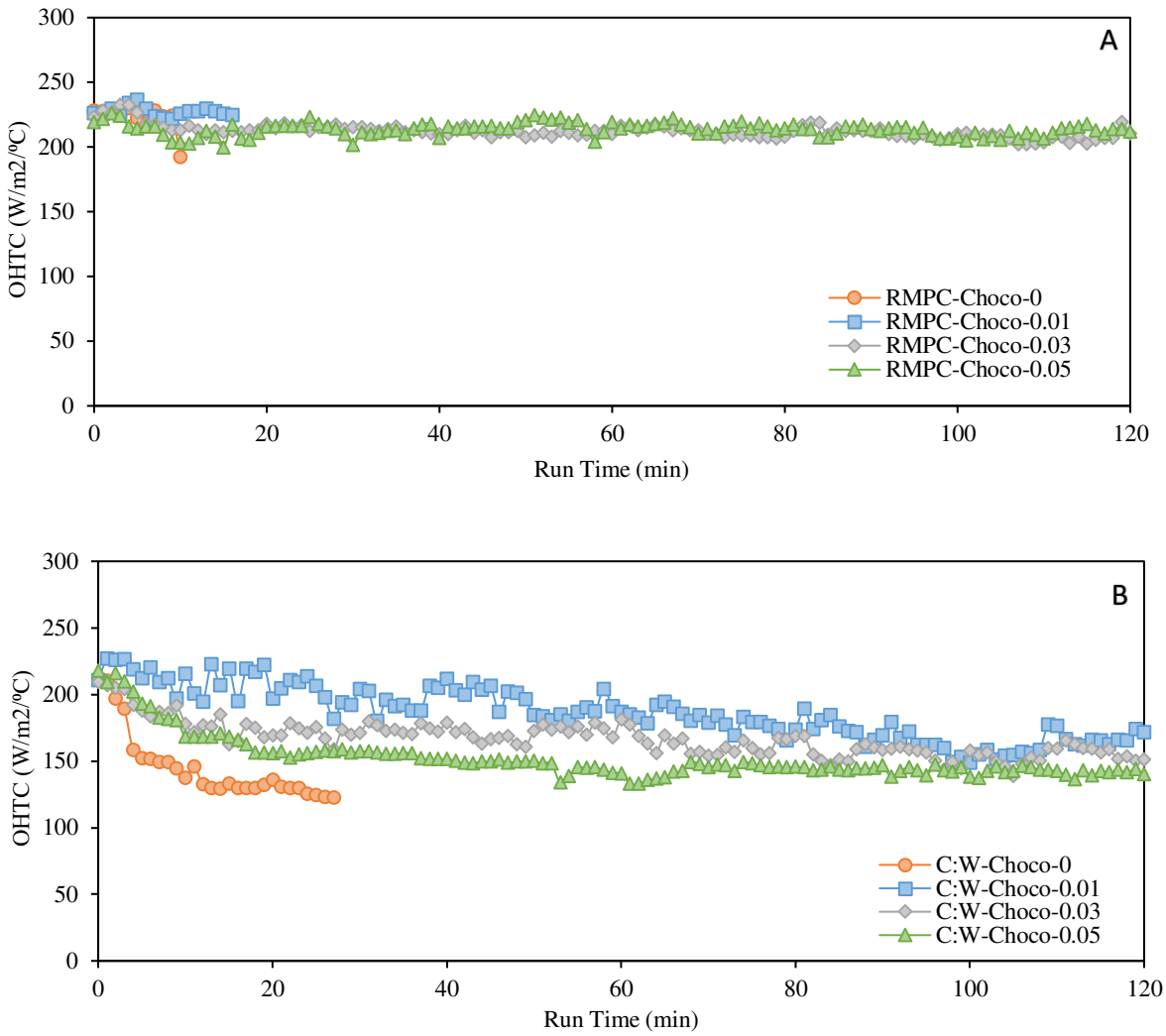

Figure 6.2: Variation in OHTC with run time for chocolate flavoured high protein beverages (A) RMPC-Choco samples (B) C:W samples. Average data of duplicate runs is presented here.

Settlement of cocoa particles in the processing line could be responsible for the low UHT stability of RMPC-Choco-0 and C:W-Choco-0, which resulted in severe fluctuations in back pressure and OHTC (Prakash et al., 2010). The improvement in UHT stability of chocolate beverage formulations by addition of κ-carrageenan can be related to a three dimensional network and weak gel formed by electrostatic interactions between sulphate groups of κ-carrageenan and κ-casein present on the surface of casein micelles (Spagnuolo, Dalgleish, Goff, & Morris, 2005). Similarly, whey proteins and κ-carrageenan interactions are also reported to be electrostatic in nature, contributing to the formation of a weak gel (Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004). This weak gel helped to entrap large cocoa particles and reduced their settlement onto processing line decreasing surface fouling.

Very low levels of κ-carrageenan are required to induce gelation in the presence of milk proteins (Drohan, Tziboula, McNulty, & Horne, 1997). Therefore, it is necessary to find a critical κ-

carrageenan concentration to form a weak gel that can hold cocoa particles in suspension in the beverage mix. Exceeding this critical concentration may cause loss of fluidity of a beverage due to increase in viscosity. This critical concentration of κ-carrageenan for RMPC-Choco and C:W-Choco was found to be 0.03 and 0.01%, respectively. Increasing the κ-carrageenan concentration up to 0.05% did not adversely affect the UHT stability of samples, however there was a notable change in the fluidity of samples, which will be discussed in details in the rheological and particle size analysis sections.

C:W-Choco required lower amount of κ-carrageenan for its improved UHT stability as compared to RMPC-Choco, which could be attributed to a higher concentration of whey proteins present in C:W-Choco samples. Whey proteins can form gels during thermal processing, due to heat induced denaturation and aggregation. Additionally, whey proteins also associate with casein micelles through β-lactoglobulin (β-lg) and κ-casein interactions (Donato & Guyomarc'h, 2009). Formation of higher amount of whey protein gel in C:W-Choco in addition to κ-carrageenan-milk protein interactions could be responsible for requirement of lower amount of κ-carrageenan to reduce UHT fouling (Goh, Sarkar, & Singh, 2014), where cocoa particles were immobilised by both whey protein formed gel network and milk protein-hydrocolloid gel network.

6.3.2 Particle size distribution

An increase in the amount of κ-carrageenan caused an increase in the average particle size of UHT processed samples (Fig 6.3), which was more pronounced in case of C:W-Choco samples as compared to RMPC-Choco samples. The increase in particle size on the addition of κ-carrageenan was an indication of the formation of micron size gel particles which could help in suspending the cocoa particles in the beverage mix imparting stability against sediment formation during UHT processing (Boomgaard et al., 1987). However increasing the κ-carrageenan content beyond its critical concentration for C:W-Choco was shown to decrease OHTC values, indicating formation of a fouling layer due to settlement of larger particles on heat exchanger surface. The gel formation and gel strength in milk protein-hydrocolloid system depends upon several factors and one of them is gelation temperature, above which the gel sets in and entrap the cocoa particles (Goh et al., 2014; Syrbe, Bauer, & Klostermeyer, 1998). Another important factor is the type of milk protein present in the system, such as the presence of higher amounts of whey proteins in C:W-Choco samples (Syrbe et al., 1998), which undergo denaturation and aggregation during UHT processing increasing average particle size distribution of samples (Table 6.2). The particle size distribution of RMPC-Choco-0.01 was similar to that of RMPC-Choco-0, corresponding to the similarity in UHT run time and OHTC

decrease in both the samples. While the average particle size in C:W-Choco-0.03 and C:W-Choco-0.05 was much bigger than other samples corresponding to their gel-like behaviour.

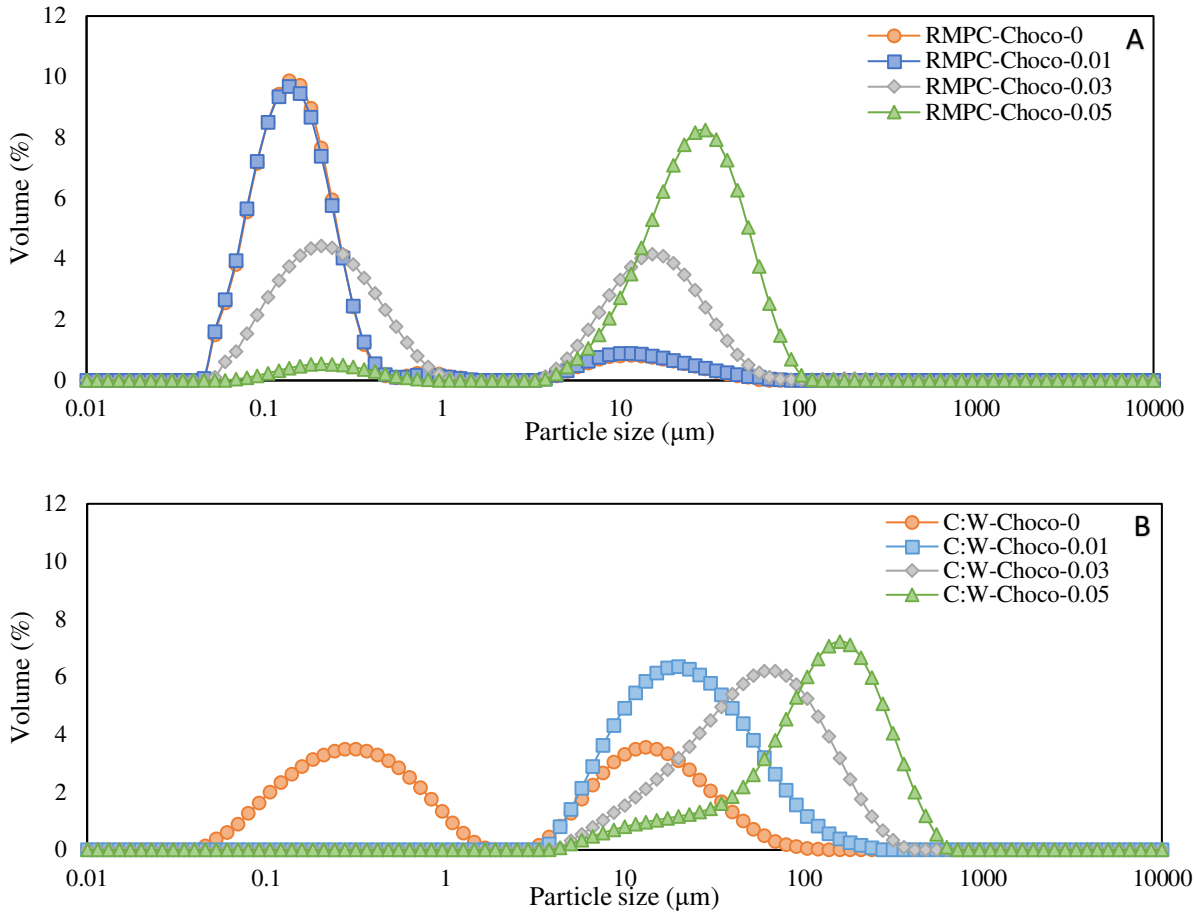

Figure 6.3: Particle size distribution of UHT processed chocolate flavoured high protein beverages (A) RMPC-Choco samples (B) C:W-Choco samples. Average data of four measurements.

Table 6.2: Comparison of volume weighted mean diameter, surface weighted mean diameter and particle size distribution of UHT processed chocolate flavoured high protein beverages.

Sample	D(4,3) (μm)	D(3,2) (μm)	D(0.1) (μm)	D(0.5) (μm)	D(0.9) (μm)
RMPC-Choco-0	1.58 ± 0.06^{d}	0.13 ± 0.00^{c}	0.07 ± 0.00^{c}	0.14 ± 0.00^{d}	1.08 ± 0.34^{d}
RMPC-Choco-0.01	1.80 ± 0.04^{d}	0.13 ± 0.00^{c}	0.07 ± 0.00^{c}	0.14 ± 0.00^{d}	5.19 ± 0.01^{d}
RMPC-Choco-0.03	8.82 ± 1.92^{cd}	0.31 ± 0.03^{c}	0.11 ± 0.01^{c}	0.57 ± 0.19^{d}	23.69 ± 2.06^{cd}
RMPC-Choco-0.05	26.72 ± 2.88^{cd}	10.74 ± 13.02^{c}	5.52 ± 7.20^{bc}	23.49 ± 2.48^{c}	50.37 ± 2.20^{cd}
C:W-Choco-0	8.10 ± 2.43^{cd}	0.36 ± 0.06^{c}	0.13 ± 0.02^{c}	2.28 ± 2.36^{cd}	23.49 ± 5.76^{cd}
C:W-Choco-0.01	28.34 ± 1.42^{c}	15.28 ± 0.04^{bc}	7.30 ± 0.21^{bc}	19.79 ± 0.37^{cd}	59.55 ± 5.27^{c}
C:W-Choco-0.03	64.70 ± 13.89^{b}	31.15 ± 3.48^{b}	13.64 ± 0.68^{ab}	50.98 ± 12.01^{b}	134.78 ± 29.96^{b}
C:W-Choco-0.05	140.71 ± 11.21^{a}	55.43 ± 3.37^{a}	24.08 ± 1.91^{a}	120.51 ± 10.22^{a}	282.63 ± 21.81^{a}

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different ($P<0.05$).

6.3.3 Rheological behaviour

The apparent viscosity of UHT processed RMPC-Choco and C:W-Choco samples generally increased with increasing their κ-carrageenan content (Fig 6.4A and B). Addition of 0.01% κ-carrageenan did not affect the viscosity of RMPC-Choco significantly and this sample was not UHT stable, similar to the control sample. However further increase in κ-carrageenan content increased the viscosity of samples significantly and consequently those samples were stable to UHT processing. Additionally, viscosities of all κ-carrageenan added C:W-Choco were significantly higher than their control sample. This again suggested the requirement of a threshold amount of κ-carrageenan in order to UHT successfully process a chocolate flavoured high protein beverage. This increase in viscosity was due to the formation of κ-carrageenan and milk protein gel network (Yanes, Durán, & Costell, 2002a) which reduced the settlement of cocoa particles on the heat exchanger surfaces and extended the UHT run-time (Prakash et al., 2010). The viscosity results corresponded well to the particle size distribution trends.

All UHT processed samples showed a non-Newtonian behaviour (Fig 6.4B), which became more pronounced with an increase in hydrocolloid (κ-carrageenan) content as also observed by (Yanes et al., 2002a). The flow behaviour index (n) (Fig 6.4C) of samples was calculated by fitting rheology

data to power law model which showed that the higher the amount of κ-carrageenan in the sample was the higher the departure of the flow behaviour of sample away from Newtonian type flow was. It can also be observed that C:W-Choco based samples showed more pronounced shear thinning behaviour and their values of flow behaviour index were lower than that of similar RMPC-Choco samples. This can be again related to the high concentrations of whey proteins in C:W-Choco samples (Tang, 1993) and also reported in our previous work of Chapter 5.

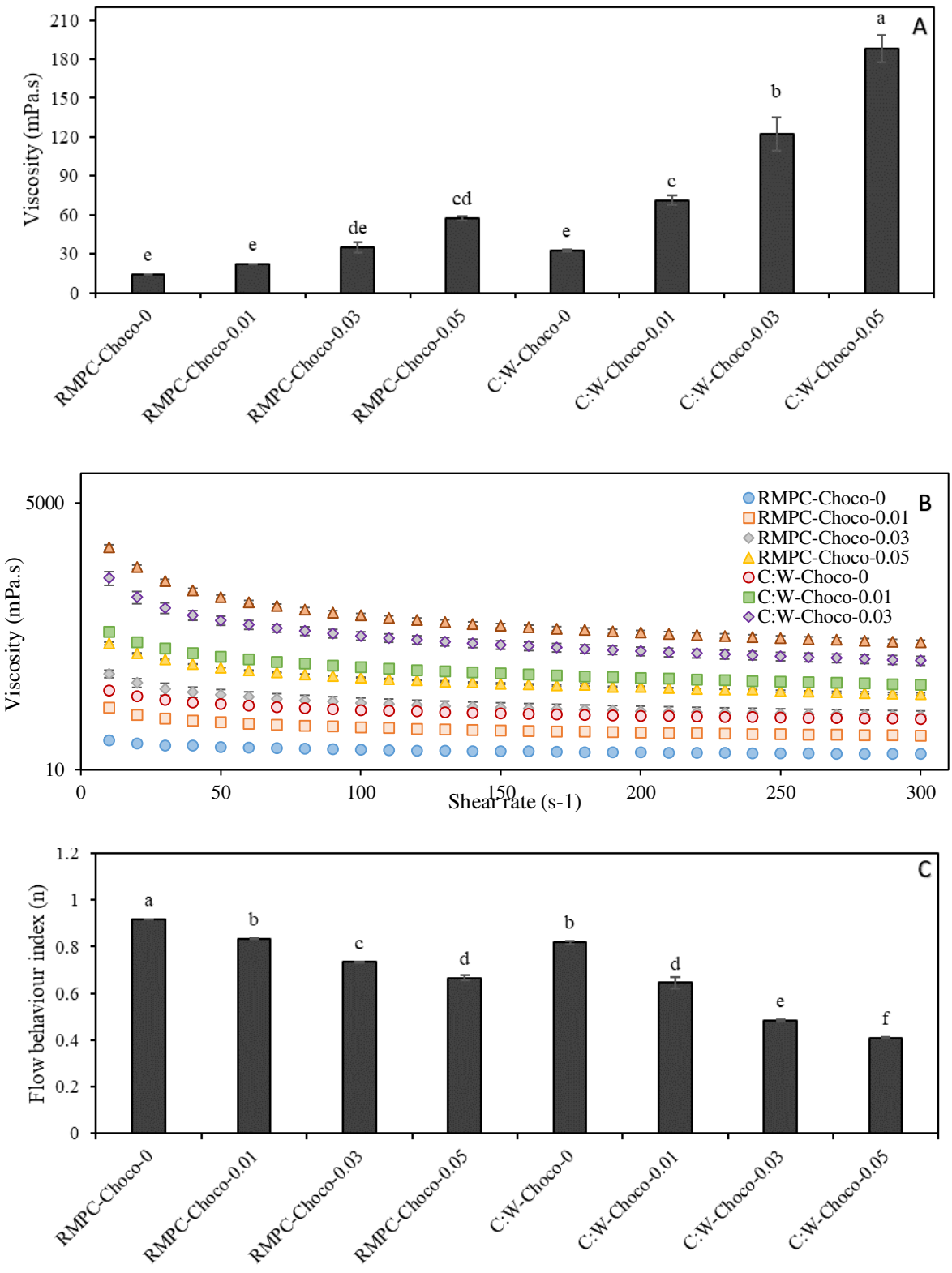

Figure 6.4: Rheological properties of chocolate flavoured high protein beverages (A) Apparent viscosity at 300 s^{-1} shear rate, unheated vs UHT processed (B) viscosity of UHT treated samples as a function of shear rate (C) Flow behaviour index (n) of UHT processed milk protein dispersions. Error bars represent standard deviation, n=4. Means with different superscripts are significantly different (P<0.05).

6.3.4 Sedimentation behaviour

Quantitative sedimentation data was not available for UHT processed C:W-Choco-0.03 and C:W-Choco-0.05 samples due to their gel like behaviour and difficulty in centrifugation. Among the remaining six samples, there was no significant difference found in the amount of sediment in five samples, except for C:W-Chcoc-0.01 (Fig 6.5). There was a possibility of a part of these sediments to be formed by milk proteins in addition to cocoa particles (the protein content contribution to sample formulations from cocoa powder and κ-carrageenan was negligible) as sedimentation measurement method used in this study is similar to the method to quantify sedimentation (large protein aggregates) in UHT milk (Boumpa, Tsioulpas, Grandison, & Lewis, 2008). Therefore, the amount of non-protein sediments was also calculated, which showed that non-protein sediment amount in C:W-Chcoc-0.01 was only significantly higher than RMPC-Choco-0.03. However, a large amount of C:W-Choco-0.01 sediment was formed from milk protein aggregates as compared to other samples (data not shown) and the gel network formed by aggregates could have entrapped higher amounts of cocoa particles. These results also showed that sedimentation data obtained by centrifugation cannot be reliably used to predict UHT stability of a chocolate flavoured milk beverage as it did not correlate with UHT stability (Fig 6.1 and 6.2) and amount of κ-carrageenan present in the samples.

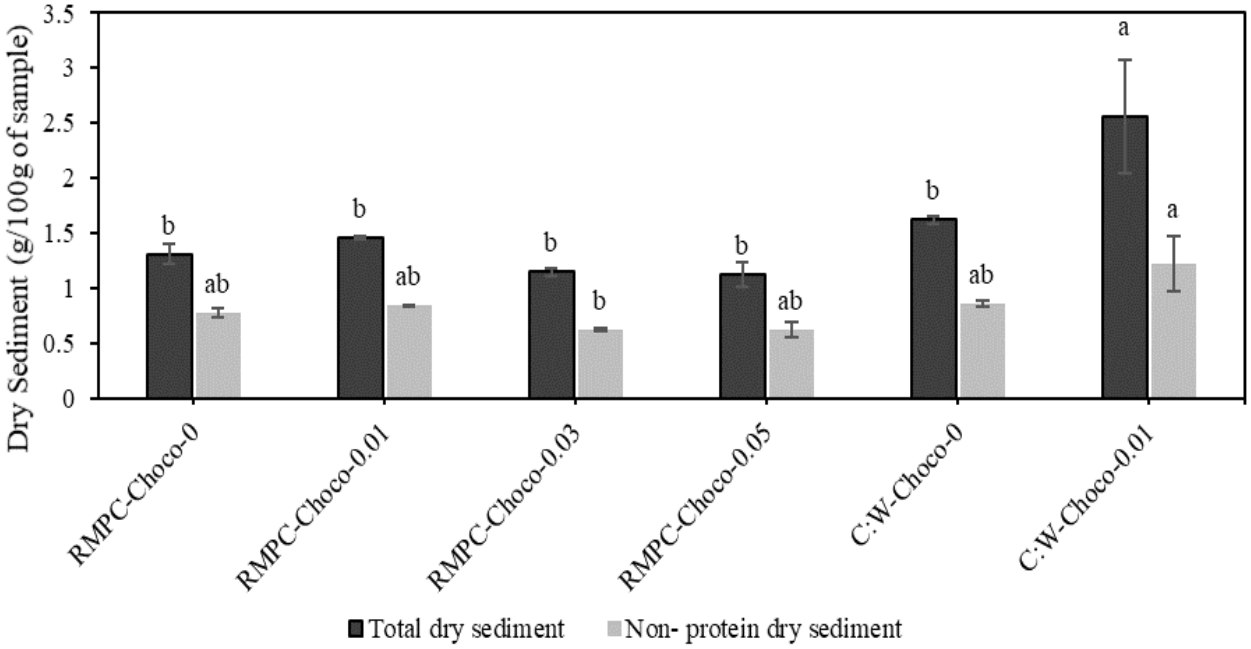

Figure 6.5: Amount of sediment formation on centrifugation (3000*g* for 15 min) in chocolate flavoured high protein beverages. Error bars represent standard deviation, n=2. Means in a single column with different superscripts are significantly different (P<0.05).

Visual observation of samples showed that a darker layer was formed by the settlement of larger cocoa particles at the bottom of containers in all unprocessed samples, which changed in thickness or disappeared after UHT processing depending upon κ-carrageen and whey protein concentration (Fig 6.6). This showed that the UHT processing temperatures not only commercially sterilised samples but also helped to induce gel formation by κ-carrageen-milk protein interactions depending upon κ-carrageen concentration and type of milk proteins (Sedlmeyer & Kulozik, 2006; Syrbe et al., 1998).

Figure 6.6: Visual observation of sediment formation under the influence of gravity in chocolate flavoured high protein beverages. In each pair left and right tubes contain unheated and UHT samples, respectively. *UHT sample formed a gel.

6.3.5 Sensory evaluation of chocolate flavoured high protein beverages and its relationship with their physical properties

Based on UHT stability and physical properties of all the samples, RMPC-Choco-0.03, RMPC-Choco-0.05 and C:W-Choco-0.01 were selected for sensory evaluation by a panel of untrained volunteers. Samples were ranked 1, 2 or 3 based on different sensory attributes. Sample with the highest perception of a sensory attribute was ranked 1 and sample with lowest perception was ranked 3. Table 6.3 provides rank sums of these three samples for all sensory attributes analysed.

Table 6.3: Rank sums for sensory attributes of UHT processed chocolate flavoured high protein beverages.

Sensory Attribute	RMPC-Choco-0.03	RMPC-Choco-0.05	C:W-Choco-0.01
Appearance (visual appeal, colour)	95^{b}	96^{b}	49^{a}
Thickness	113^{c}	79^{b}	48^{a}
Smoothness	68^{a}	78^{ab}	94^{b}
Creaminess	94^{b}	77^{ab}	69^{a}
Residual coating	96^{b}	86^{b}	58^{a}
Astringency	101^{c}	80^{b}	59^{a}
Overall preference	77^{a}	77^{a}	86^{a}

Sample with a different letter for a particular attribute is significantly different ($P<0.05$)) from other samples

C:W-Choco-0.01 sample was found more visually appealing by panellists as compared to the other two samples. It could be related to the different formulation of this sample (MPC and WPC mix as compared to MPC only of RMPC-Choco samples), which imparts a different colour profile (Table 6.4). As it can be observed that both yellowness and redness of C:W-Choco-0.01 were significantly higher than RMPC-Choco samples, whereas there was no significant difference in the lightness of samples (Table 6.4). If given a choice, consumers generally show preference to foods with more yellow or red colours as described by Williams (1992) and Clydesdale (1993).

Table 6.4: Hunter L, a and b values of cheese obtained by colorimeter of UHT processed chocolate flavoured high protein beverages.

Sample	L	a	b
RMPC-Choco-0.03	36.89±0.28[a]	3.33±0.04[b]	2.55±0.19[b]
RMPC-Choco-0.05	35.54±0.57[a]	2.99±0.57[c]	2.72±0.25[b]
C:W-Choco-0.01	34.72±1.59[a]	5.40±0.04[a]	5.54±0.04[a]

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different (P<0.05).

Sensory panel's assessment of the thickness of samples was similar to instrumental apparent viscosity data of samples (Fig 6.4A) as this sensory attribute is related to the flow behaviour of fluid and the force required to press the sample between the tongue and palate (Ningtyas et al., 2019). Smoothness and creaminess perception of samples were contrary to each other. Lower smoothness of RMPC-Choco-0.03 can be related to smaller average particle size (Table 6.2) and lower viscosity of this sample (Fig 6.4A), which could have led to ease in the formation of a thin layer between tongue and palate (Van Aken, Vingerhoeds, & de Hoog, 2007). Panellists reported higher creaminess in C:W-Choco-0.01 sample, similar to thickness results. The creaminess is the combined perception of fat, smoothness and viscosity (Ningtyas et al., 2019), so this suggests that viscosity or thickness of samples dominated the smoothness factor in panellist's perception of creaminess. Possibly the higher viscosity of C:W-Choco-0.01 sample resulted in a sensation of softness and creaminess shadowing any graininess that could have been caused by larger particle sizes (Garrec & Norton, 2013).

The residual coating was reported higher for C:W-Choco-0.01 sample, which was again due to larger average particle size and higher viscosity of this sample. Larger particles are more likely to interact and physically entangle within the irregularities of the tongue surface (Van Aken et al., 2007). C:W-Choco-0.01 also showed higher astringency, which is commonly related to whey protein beverages (Beecher et al., 2008b) and this sample contained higher amounts of whey proteins as compared to RMPC-Choco samples. Finally, there was no significant difference found for overall preference by the panellists for these three sample, which could be a result of a difference in individual preferences of different panellists for a chocolate flavoured drink.

6.4 Conclusions

This work demonstrated that κ-carrageenan requirement in order to prepare UHT stable chocolate flavoured high protein beverages was influenced by their protein composition. Presence of higher amounts of whey proteins in C:W-Choco lowered their minimum threshold requirement of κ-carrageenan as compared to RMPC-Choco due to entrapment of cocoa particles by whey protein aggregates along with milk-protein κ-carrageenan network. The sensory attributes of these two types of chocolate flavoured high protein beverages were also influenced by their protein composition. The physical properties measured could be related to the sensory attributes' perception by an untrained sensory panel. For example, higher viscosity of C:W-Choco-0.01 as compared to RMPC-Choco samples was also perceived by sensory panellists as the higher thickness of this sample. The results from this study can help in the formulation and development of commercial chocolate flavoured high protein beverage.

CHAPTER 7: ULTRA HIGH TEMPERATURE (UHT) STABILITY OF HIGH PROTEIN DISPERSIONS PREPARED FROM MILK PROTEIN-SOY PROTEIN HYDROLYSATE MIXTURES

The following submitted manuscript has been incorporated as Chapter 7:

Singh, J., Prakash, S., Bhandari, B., & Bansal, N. (Submitted in October, 2019). Ultra high temperature (UHT) stability of high protein dispersions prepared from milk protein-soy protein hydrolysate mixtures, under review in LWT Food Science and Technology.

Contributor	**Statement of contribution**
Jaspal Singh (Candidate)	Concept and design (85%) Analysis and interpretation (70%) Drafting and production (55%)
Sangeeta Prakash	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (10%)
Bhesh Bhandari	Concept and design (5%) Analysis and interpretation (5%) Drafting and production (10%)
Nidhi Bansal	Concept and design (5%) Analysis and interpretation (20%) Drafting and production (25%)

Abstract:

The objective of this study was to investigate the effect of the addition of soy protein hydrolysates on UHT stability of milk protein beverages at high protein content. Also, there is a lack of reported data on UHT stability of soy protein hydrolysates. UHT stability of 8% protein reconstituted milk protein concentrate (8-RMPC), 8% protein soy protein hydrolysate (8-RSPH) and 8-RMPC with added 1, 2 and 3% protein RSPH (e.g. 8-RMPC+1-RSPH) was studied. Both 8-RMPC and 8-RSPH showed very high UHT stability (UHT plant run-time > 120 min). Inclusion of 1-RSPH in 8-RMPC did not affect UHT run-time (> 120 min) and overall heat transfer coefficient (OHTC). A significant drop in OHTC was observed in 8-RMPC+2-RSPH without reducing the UHT run-time below 120 min mark and required UHT processing temperatures could be maintained (143-146 ºC) throughout the UHT run. 8-RMPC+3-RSPH showed markedly reduced UHT stability, both shorter UHT run-time (61 min) and very low OHTC values were observed which can be attributed to the formation of larger protein aggregates (D(4,3) was 7.03 and 0.46 µm for 8-RSMP+3-RSPH and 8-RSMP+2-RSPH, respectively). An increase in apparent viscosity (42.03 and 11.83 mPa.s for 8-RSMP+3-RSPH and 8-RSMP+2-RSPH, respectively) of UHT processed samples would have also induced severe fouling during UHT.

7.1 Introduction

Incorporation of high quality proteins in the human diet is positively related to muscle gain, losing weight and controlling diabetes (Etzel, 2004). Additionally, peptides derived from hydrolysis of dietary proteins are often shown to have antihypertensive, anti-inflammatory, immunomodulatory, antioxidant, antimicrobial, hormonal regulating and various other bioactive functions in human body (Maestri, Marmiroli, & Marmiroli, 2016; Nielsen, Beverly, Qu, & Dallas, 2017). There has been a recent trend of increased interest in the incorporation of peptide ingredients in food product formulation due to their bioactivity (Li-Chan, 2015). Apart from health benefits, researchers have also shown that controlled hydrolysis of proteins can enhance their functional properties; such as solubility, foaming, gelling and emulsifying properties when compared to native proteins (Barać, Stanojević, Jovanović, & Pešić, 2004).

High protein beverages are a convenient source of protein to meet a large amount of daily recommended protein intake. These products are commonly manufactured using milk proteins and to a lesser extent plant proteins (Sethi, Tyagi, & Anurag, 2016). Growth in the consumption of plant proteins and plant protein fortified beverages has also been observed (Lan et al., 2018). Soy protein is arguably one of the most popular plant based protein used in the food industry (Liu, 1997). Soybean is a significant source of dietary protein and soy proteins, similar to milk proteins contain all of the essential amino acids vital for human nutrition (Liu, 1997).

Soy protein based beverages, however are not a very popular consumer choice as compared to dairy protein based beverages in western countries, owing to their undesirable sensory properties (Beliciu & Moraru, 2011). There has been several studies carried out on the addition of low amounts of soy proteins in dairy products such as low fat ice-cream and yogurt (Biswas, Chakraborty, & Choudhuri, 2002; Friedeck, Aragul-Yuceer, & Drake, 2003). Similarly, small but dietary significant amounts of soy protein ingredients can be added to high milk protein beverages to increase their consumer acceptance (Singh, Kumar, Sabapathy, & Bawa, 2008). Soy protein fortified high milk protein beverages can be an easy way for consumers to incorporate more soy proteins into their diet.

In the present work milk protein concentrate (MPC) was used as the main ingredient for formulating milk protein-soy protein dispersions, a decision based on the knowledge gained from our previous work showing high heat stability of reconstituted MPC (RMPC) (Chapter 3). The protein content in different types of MPCs varies from approximately 50-85% per total solids and they contain complete milk protein system in the same ratio of casein and whey proteins as found in cow's milk (Havea, 2006; Yanjun et al., 2014). This makes MPC an excellent form of concentrated milk protein ingredient, which can be used to formulate neutral pH (pH ~ 6.8) high protein beverages (Banach,

Lin, & Lamsal, 2013). Soy protein ingredients are available as soy protein concentrate, soy protein isolate and soy protein hydrolysate. Hydrolysed soy protein isolates have superior solubility as compared to non-hydrolysed soy protein isolates (Ortiz & Wagner, 2002; Panyam & Kilara, 1996). Solubility is considered as one of the most important factors when selecting a protein ingredient for beverage mix (Jideani, 2011; Phillips, Whitehead, & Kinsella, 1994).

Ultra high temperature (UHT) technology is a preferred mode of processing neutral pH high protein beverages to produce a commercially sterilize shelf-stable product with minimal thermal damage to nutrients and sensory attributes (Beecher et al., 2008a; Burton, 1994). However, there are concerns about the thermal stability of protein ingredients during UHT processing, which can cause the formation of deposit layers on heat exchanger surfaces. This fouling of surfaces results in decreased processing plant run times, requiring frequent down times for cleaning in place operations (Burton, 1994). Our previous work on the heat stability of MPC showed that milk protein dispersions formulated using MPC85 were very stable during UHT processing. RMPC at high protein content (7.5 to 14% w/w) showed very less signs of heat exchanger surface fouling and could be UHT processed effortlessly leading to long run-times of UHT plant (Chapter 3).

Most of the research work on the UHT processing of soy products has been carried out on soy milk (Durand et al., 2003; Kwok, Liang, & Niranjan, 2002), e.g. Pathomrungsiyounggul, Grandison, and Lewis (2012); Pathomrungsiyounggul, Grandison, and Lewis (2007); Pathomrungsiyounggul, Grandison, and Lewis (2010); Pathomrungsiyounggul, Lewis, and Grandison (2010) carried out detailed studies on the effect of thermal processing, calcium fortification and calcium chelating salts on the physicochemical properties of soymilk. There is a lack of reported data on UHT stability of soy protein ingredients and their compatibility with milk proteins during thermal processing. The present work addressed this issue and was designed to gain insights into UHT stability and fouling behaviour of milk protein-soy protein hydrolysate mixtures. This knowledge can be helpful in the formulation of UHT-stable dairy based high protein dispersions with added soy peptides for various food product applications.

7.2 Materials and Methods

7.2.1 Materials

Commercially manufactured MPC was purchased from Maxum Foods Pty. Ltd, Australia. A commercial sample of soy protein hydrolysate (SPH) were obtained from Nutrily Biotechnology Ltd., People's Republic of China. MPC and SPH contained on average 81.5 and 86.0% protein, respectively, as per the compositional data provided by the suppliers. The SPH used in this study was

extensively hydrolysed (with a degree of hydrolysis of 75%) and comprised mostly of soy peptides, with narrow molecular weight distribution. The molecular weight distribution of 85.59% and 12.12% of peptides was between 186-1000 Da and 1000-2000 Da, respectively, with an average molecular weight of 720 Da and consisted of 3-5 amino acids. A complete description and protein profile analysis of this SPH ingredient can be found in Zhao et al. (2018).

7.2.2 Preparation of protein dispersions

A calculated amount of protein powders were added to distilled water heated to 50±2 °C to achieve required (w/w) protein content in the reconstituted sample. The samples were kept refrigerated (3-4 °C) overnight (~ 14 h) to allow complete hydration of powder particles. The pH of samples was analysed after rehydration and adjusted to 6.8 at room temperature, using 2M NaOH or 2M HCl. Table 7.1 shows different samples and sample codes used in this work. Suffix Unheated and UHT were used to denote unheated and UHT heated samples, respectively. Milk protein dispersions were filtered to remove any undissolved particles (almost negligible).

Table 7.1: Description of reconstituted protein dispersions used

Sample Name	Ingredients	Remarks
X-RSPH	Reconstituted SPH	'X' denotes protein content in the sample coming from MPC or SPH (ranging from 2 to 8% for MPC and 1 to 8% for SPH)
X-RMPC	Reconstituted MPC	
X-RMPC+X-RSP	RMPC and RSPH mixed together	

7.2.3 Heat coagulation time measurements

Heat coagulation time (HCT) as measured using the method described by Davies and White (1966) with some modifications. The HCT temperature used was similar to UHT sterilization (145 ºC). Glass vials (22.6 x 75.5 mm) containing 2 mL sample were placed on a rocker and immersed in a temperature controlled oil bath for heating. The rocker speed was kept at ~8 revolutions per min. HCT was reported as time elapsed between putting the samples in the oil bath and appearance of first visible signs of coagulation (as described in Chapter 3). All experiments were performed in duplicate.

7.2.4 Ionic Ca activity in reconstituted milk protein dispersions

Ca-ion activity in protein dispersions was measured using LAQUAtwin calcium ion meter (Horiba Instruments, Japan). The calcium ion meter was calibrated using 3.74 mM (150 ppm) and 49.90 mM

(2000 ppm) Ca-ion activity standard solution before each experiment, according to manufacturer instructions. All measurements were performed in duplicate at room temperature.

7.2.5 Measurement of UHT stability and fouling behaviour of protein dispersions

The samples were UHT processed using a bench-top UHT plant. The description and flow diagram of the plant are given in Chapter 3. The samples was pre-heated to 95 °C for 8 s and sterilization temperature was 145 °C for 5 s. The volumetric flow rate of the sample in the beginning of the trial was kept at 120 mL/min ($2X10^{-6}$ m^3/s). All experiments were performed in duplicate and the results presented are the average of the two runs. The following indicators were used to detect signs of fouling and UHT processing was stopped to avoid complete blockage of UHT plant: (i) The back pressure could not be maintained at 0.4 MPa and high back pressure triggered the over pressure valve, (ii) the outlet temperature of sterilization section dropped below 120 ºC and (iii) blockage of product channel due to severe fouling. Unless otherwise stated, if none of the above factors stopped UHT processing, the experiment was terminated after 120 min has elapsed into the UHT run. Rate of fouling during UHT run was measured using the drop in overall heat transfer co-efficient as described in Chapter 3.

7.2.6 Particle size distribution of protein dispersions

A Malvern Mastersizer 2000MU-A (Malvern Instruments Ltd, Malvern, United Kingdom) was used to analyze particle size distribution (PSD) by dynamic light scattering (DLS) as described by Dumpler and Kulozik (2016). The refractive index of protein was set at 1.41 and for dispersant (distilled water) was 1.33. Particle absorption index was kept as 0.001. Stirrer speed was set at 2000 rpm and laser obscuration was maintained between 10 and 11 during measurement. All measurements were performed at room temperature (23 °C).

7.2.7 Rheological analysis

An AR-G2 Rheometer (TA Instruments Ltd., USA) equipped with a 60 mm parallel plate geometry was used to collect rheological data of unheated and UHT processed samples. Sample temperature was kept at 20 ºC on a Peltier plate and the gap between the Peltier plate and parallel plate geometry was kept at 300 μm during measurements. Measurements were performed after samples were allowed temperature equilibration for 1 min. Steady shear rate sweep test was performed on samples at shear rates from 10 to 300 s^{-1}. Power Law model was used to calculate flow behavior index (n) as shown in the equation (1) below (Ibarz, Giner, Pagan, Gimeno, & Garza, 1995a):

$$\sigma = K\gamma^n \qquad \text{eq. 7.1}$$

Where, σ is the shear rate (Pa.s), K is the consistency co-efficient (Pa.s^n), γ is the shear rate (s^{-1}), and n is the flow behavior index.

7.2.8 Optical microscopy

A small amount of sample was placed on a microscopy slide and photographed under an optical microscope (Leica DM 2500, Leica Microsystems, Australia) at a magnification of 100× to complement particle size distribution data. A Leica DFC450 C digital camera (Leica Microsystems, Australia) was used to capture images.

7.2.9 Colour measurements

The color measurements on samples were carried out using a Minolta Konica Chroma Meter CR-400 (Konica Minolta, INC, Japan). Hunter *L, a* and *b* values for color parameters were recorded. A white tile (Y = 94.93, x = 0.3131, y = 0.3197) was used to calibrate the instrument before sample measurements.

7.2.10 Gel electrophoresis

Non reducing tricine sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) analysis was performed using precast polyacrylamide gels (12%) according to the method described by Haider, Reid, and Sharp (2012). A Precision Plus Protein™ Dual Xtra molecular weight standard (Bio-Rad Laboratories Pty. Ltd, Australia) was used to identify protein bands. Samples were mixed 1:1 with 2X sample buffer before loading them to the gel. 10 µg protein was loaded in each well. Electrophoresis was carried out at 100 V until the end of the run using a Bio-Rad Mini Protean Tetra Cell system (Bio-Rad Laboratories Pty. Ltd, Australia). After destaining the gels were scanned and analyzed using a Bio-Rad GS-800 calibrated Densitometer (Bio-Rad Laboratories Pty. Ltd, NSW, Australia).

7.2.11 Statistical analysis

The data obtained from all experiments was analysed using Microsoft Excel, SigmaPlot and Minitab 16 software package. Significant differences between average values of replicate measurements at each data point was analysed by analysis of variance (ANOVA) using Tukey's HSD post hoc test at 95% confidence level.

7.3 Results and discussion

7.3.1 Assessment of heat stability of soy protein hydrolysate and its compatibility with milk proteins

In this study, SPH was chosen over soy protein concentrate or isolate due to its superior solubility and higher perceived health benefits of its bioactive peptides (Liu & Scotland, 2017). During preliminary trials, a reconstituted soy protein isolate (RSPI) showed poor solubility, larger particles as compared to RMPC and phase separation during overnight hydration. The RSPH did not show any sedimentation and there was no settlement of powder particles at the bottom even during overnight hydration step of the sample preparation procedure.

The HCT analysis (at UHT processing temperature, 145 °C) of RSPH was carried out at different protein concentrations, ranging from 2 to 8%. It was observed that RSPH had very high heat stability. The HCT analysis of all samples was terminated after 15 min had elapsed, if no coagulation of protein occurred. The HCT for RSPH at all concentrations exceeded 15 min without showing any signs of aggregate formation. The high heat stability of RSPH could be due to the fact that it was mostly constituted of small molecular weight peptides which could not form heat induced aggregates large enough to precipitate on the glass vials wall as normally observed in case of higher molecular weight milk proteins (Crowley, Dowling, Caldeo, Kelly, & O'Mahony, 2016a).

The next step was to incorporate SPH into MPC and study the heat stability of mixed protein dispersions by measuring their HCT. Preliminary HCT results showed that 50:50 ratio RSPH and RMPC mixes at different protein levels had lower heat stability as compared to both RMPC and RSPH. Therefore, to study this behaviour further and to find a suitable mix for UHT processing, HCT analysis on RMPC, with or without added 1% protein RSPH (prepared by reconstituting MPC and SPH powders together in water to obtain required RMPC and RSPH protein concentrations), at various protein contents were carried out (Fig 7.1). The heat stability of RMPC decreased gradually with an increase in its protein content, which was due to increase in heat induced aggregate formation in a concentrated system which allows more protein interactions (Le Bon et al., 1999). 2, 4 and 6% protein RMPCs with added 1% protein RSPH had markedly reduced heat stability as compared to similar protein RMPCs without added RSPH.

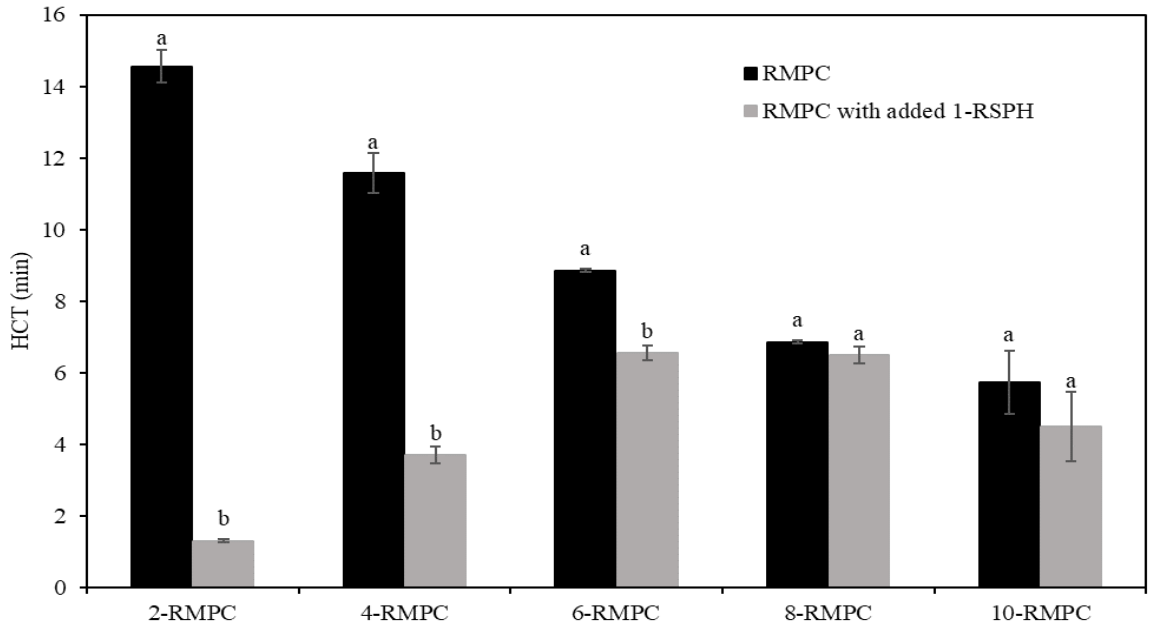

Figure 7.1: HCT of milk protein dispersions (All results are expressed as the mean ± standard deviation (n = 3)). Means for similar milk protein content of RMPC with different superscripts are significantly different (P<0.05).

These results showed that the addition of RSPH to RMPC adversely affected heat stability of RMPC, which was possibly due to the increased aggregation and coagulation of milk proteins mediated by the presence of soy peptides. Peptides can cause aggregation of intact milk proteins through hydrophobic interactions and reduction in electrostatic repulsions between proteins by binding to them during heating as observed by Creusot and Gruppen (2008). The aggregates formed in this protein-peptide system were composed of both proteins and peptides. It was also reported that peptides from whey protein hydrolysate could aggregate β-casein and whey proteins non-specifically and peptide-protein interactions were stronger when the degree of hydrolysis was increased in whey protein hydrolysate and very low molecular weight peptides (2.1 and 1.4 kDa) were present (Creusot & Gruppen, 2008).

It can be further observed that an increase in protein content of RMPC in RMPC-RSPH mix caused an increase in HCT and 8-RMPC+1-RSPH showed highest heat stability among RSPH added samples. At this protein concentration, there was no significant difference in the HCT of 8-RMPC and 8-RMPC+1-RSP. This increase in HCT with an increase in RMPC content in the protein dispersions could be possibly related to the protective effect of caseins working as chaperone proteins which controlled the heat induced protein-peptide interactions (Guyomarc'h et al., 2009). This could lead to a reduction in the formation of larger aggregates of milk proteins in the presence of a higher concentration of caseins (Chapter 5). This prevented visible coagulation of proteins during HCT test

for a longer period of time enhancing the heat stability of protein dispersions with higher amounts of RMPC. Further increase in protein content showed a decrease again in HCT of 10-RMPC+1-RSPH which corresponded to decrease in heat stability of 10-RMPC.

7.3.2 UHT stability of protein dispersions

According to HCT data 8-RMPC+1-RSPH sample showed highest heat stability, therefore 8% protein RMPC was chosen as a base material for protein dispersion mix with added RSPH to access its UHT processability. The protein content of RSPH in protein dispersion was varied from 1% (1-RSPH) to a maximum amount up to which RMPC+RSPH mix is stable to UHT processing.

RSPH showed very high heat stability in the HCT test, this was confirmed by subjecting 8% protein RSPH to UHT processing. This sample was UHT processed without any notable temperature fluctuations and pressure drops. The UHT-run was terminated after 120 min without any fouling (Fig 7.2). It has been previously reported that pure peptide solutions do not form large aggregates upon heating even when the peptide-peptide interactions are increased (Kosters, Wierenga, de Vries, & Gruppen, 2013). This could be a reasons of high UHT stability of 8-RSPH. The 8-RMPC also showed very high UHT stability and run-time exceeded 120 min without any signs of fouling, which was similar to the high UHT stability shown by high protein RMPC as reported in Chapter 3.

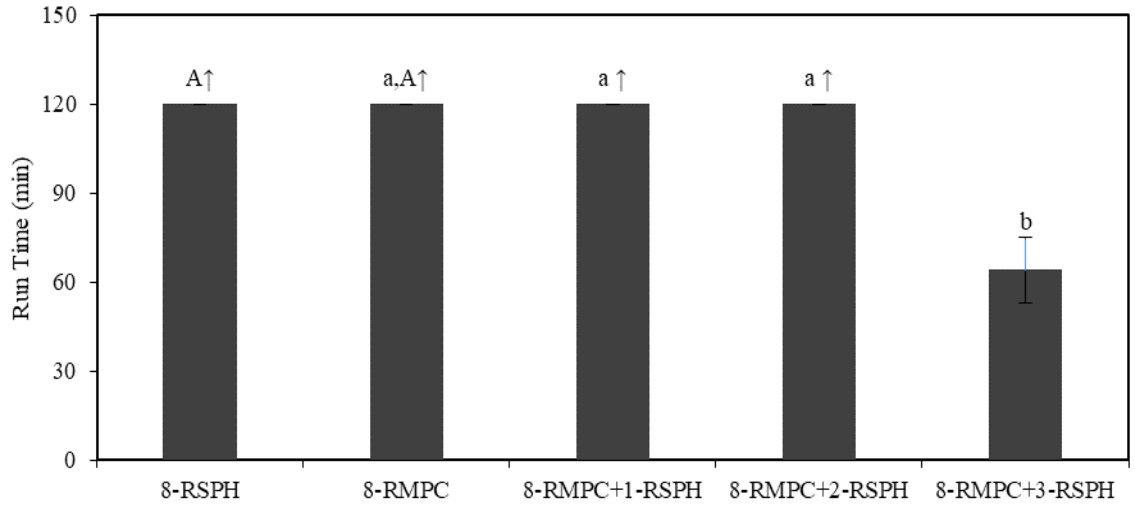

Figure 7.2: The average UHT run times on a bench top UHT tubular heat exchanger during processing of protein dispersions. Error bars represent standard deviation, n=2. Means with different letters are significantly different (P<0.05), capital letters superscripts compare 8-RSPH with 8-RMPC only. Samples with ↑did not foul in 120 min.

Further UHT runs were performed on 8-RMPC with added RSPH. 8-RMPC samples containing 1 and 2% RSPH showed high UHT stability and the UHT run for these samples exceeded 120 min. Whereas 8-RMPC+3-RSPH showed a drop in UHT run-time below the 120 min mark (UHT run-time was 64 min) along with temperature fluctuations and pressure drops. Therefore, the RSPH content in protein dispersions was not increased beyond 3% protein due to fouling and processing difficulties encountered during UHT run of 8-RMPC+3-RSPH.

The extent of fouling during UHT processing of protein dispersions can be observed as a change in OHTC during their UHT run (Fig 7.3). OHTC values were similar for 8-RSPH, 8-RMPC and 8-RMPC+1-RSPH and these three samples maintained their high OHTC throughout the entire UHT run, indicating their high UHT stability (Fig. 7.3). OHTC decreased from the beginning of the UHT run for 8-RMPC+2-RSPH, which showed that increasing the RSPH content tended to decrease UHT stability of RMPC. This drop in UHT stability of RSPH added RMPC samples became much more severe during UHT stability of 8-RMPC+3-RSPH, which showed a gradual decrease in OHTC due to excessive fouling and deposit formation. The fouling layer offered increase resistance to heat transfer which in turn lead to reduced UHT temperatures and OHTC values (Burton, 1994).

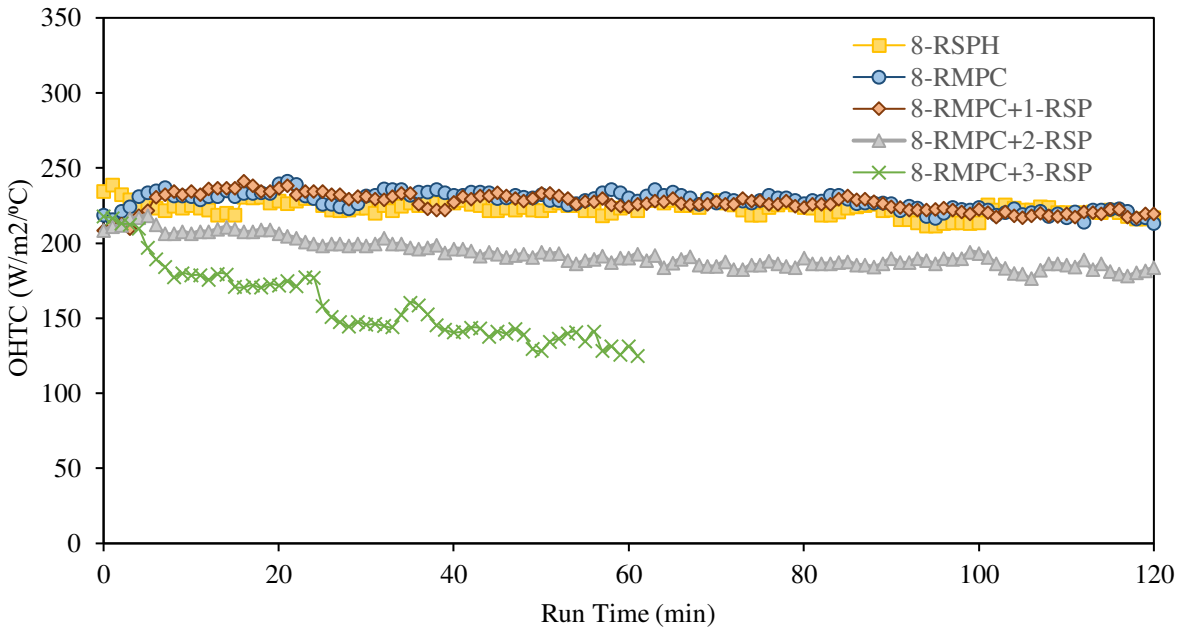

Figure 7.3: Variation in OHTC with run time for protein dispersions. Average data of duplicate runs is presented here.

7.3.3 Ionic Ca activity in reconstituted milk protein dispersions

The Ca-ion activity (Table 7.2) in 8-RSPH (0.22±0.02) was significantly lower than 8-RMPC (2.13±0.05). The addition of soy protein hydrolysate to 8-RMPC did not significantly affected its Ca-

ion activity. This suggested that reduced UHT stability of 8-RMPC+3-RSPH was not caused by any changes in its ionic calcium.

Table 7.2: Calcium ion activity in unheated milk protein dispersion samples

Sample	Calcium ion activity (mM)
8-RSPH	0.22 ± 0.02^{B}
8-RMPC	2.13 ± 0.05^{aA}
8-RMPC+1-RSPH	2.19 ± 0.10^{a}
8-RMPC+2-RSPH	2.13 ± 0.01^{a}
8-RMPC+3-RSPH	2.12 ± 0.05^{a}

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different ($P<0.05$).

7.3.4 SDS-PAGE analysis of protein dispersions

The SDS-PAGE analysis of protein dispersions before and after UHT processing is shown in Fig. 7.4. It can be observed that the protein profile of 8-RSPH remained largely unaffected by UHT processing which corroborated with its high UHT stability. There was a decrease in band intensities of β-lactoglobulin (β-lg) and α-lactalbumin (α-la) after UHT processing of 8-RMPC and RSPH added 8-RMPC samples, which indicated that denaturation and aggregation of whey proteins occurred during UHT processing (Datta et al., 2002). However, no observable difference was found in protein profiles of all 8-RMPC based samples after UHT treatment. This could be due to the nature of protein interactions during UHT processing of samples. As previously discussed soy peptides can cause aggregation of intact milk proteins through hydrophobic interactions and reduction in electrostatic repulsions between proteins (Creusot & Gruppen, 2008). Therefore SDS-PAGE results indicated that protein interactions in these samples were possibly non-covalent that were broken down by the SDS used in sample preparation for SDS-PAGE analysis (Srinivas, 2012). Therefore, possible formation of larger protein aggregates leading to an increase in fouling deposits with an increase in RSPH content were further analysed using particle size distribution and optical microscopy analysis.

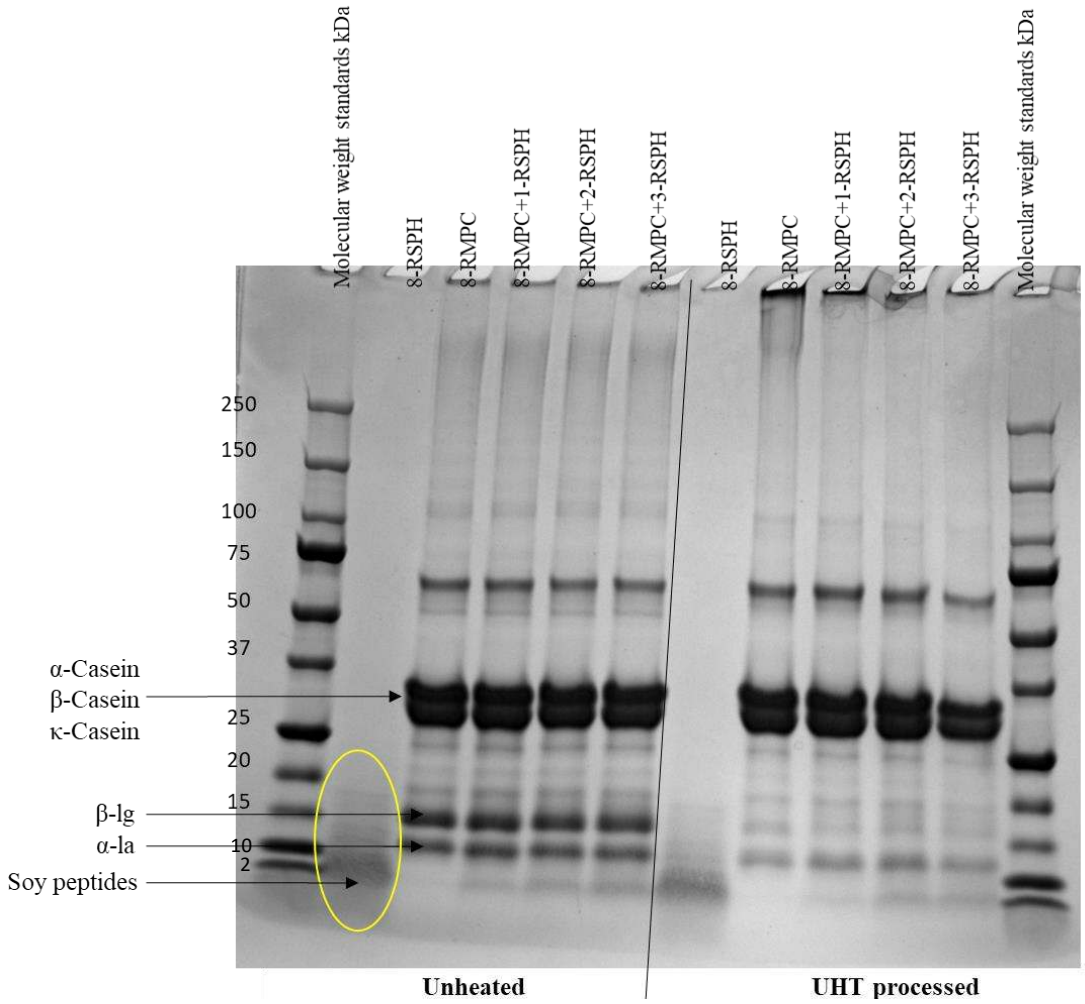

Figure 7.4: SDS-PAGE analysis of protein dispersions. Bands within the yellow oval represent soy peptides.

7.3.5 Particle size distribution of protein dispersions

The particle size distribution of unheated samples, which fell largely in the submicron range, is shown in Fig. 7.5A. The particle size distribution data of unheated and UHT processed RSPH is not available due to non-detection of any particles in these samples by the particle size analyser. RSPH contained extremely low molecular weight peptides and it was completely solubilized in water. After UHT processing, RMPC samples with different contents of RSPH showed different particle size distributions (Fig 7.5B). An increase in particle size was observed in UHT processed RMPC samples with an increase in RSPH content. 8-RMPC+3-RSPH-UHT showed that >90% of its particles were larger than 19 µm. In comparison with this sample, D(0.9) of all other UHT processed samples was of sub-micron range (Table 7.3). This correlated well with low UHT stability of 8-RMPC+3-RSPH.

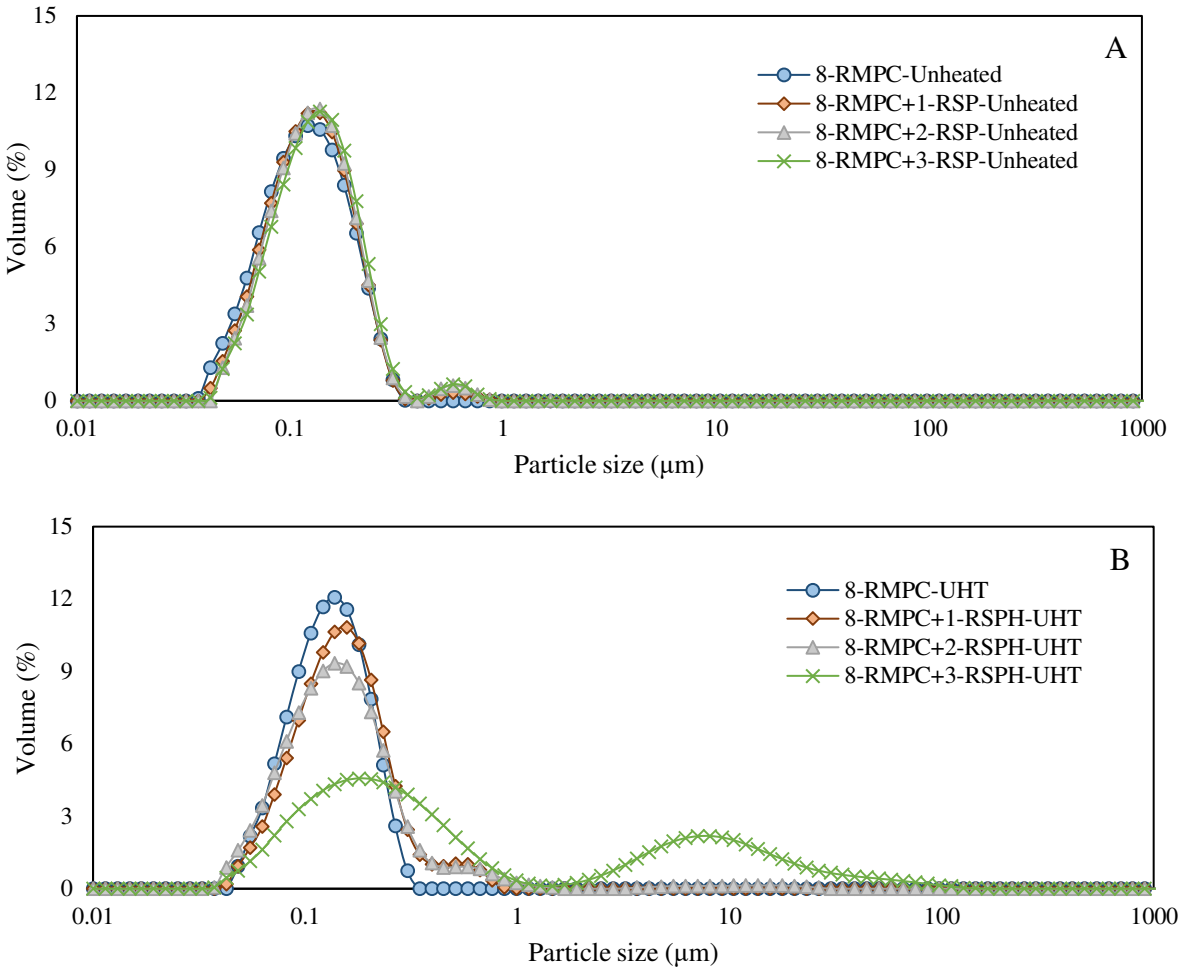

Figure 7.5: Particle size distribution of protein dispersions (A) before and (B) after UHT processing. Average data of four measurements.

Table 7.3: Comparison of volume weighted mean diameter, surface weighted mean diameter and particle size distribution of UHT processed protein dispersions.

Sample	D(4,3) (µm)	D(3,2) (µm)	D(0.1) (µm)	D(0.5) (µm)	D(0.9) (µm)
8-RMPC-UHT	0.15±0.00^{b}	0.12±0.00^{b}	0.08±0.00^{b}	0.14±0.00^{b}	0.23±0.00^{b}
8-RMPC+1-RSPH-UHT	0.19±0.03^{b}	0.14±0.01^{b}	0.08±0.00^{b}	0.16±0.02^{b}	0.31±0.08^{b}
8-RMPC+2-RSPH-UHT	0.46±0.35^{b}	0.13±0.00^{b}	0.07±0.00^{b}	0.15±0.01^{b}	0.35±0.01^{b}
8-RMPC+3-RSPH-UHT	7.03±0.24^{a}	0.24±0.01^{a}	0.10±0.00^{a}	0.36±0.04^{a}	19.00±0.64^{a}

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different ($P<0.05$).

7.3.6 Optical microscopy of protein dispersions

The optical microscopy images of unheated samples showed that a very small number of particles were formed after adding increasing quantities of RSPH to RMPC. From micrographs of UHT treated 8-RMPC and 8-RMPC+1-RSPH samples it was clear that there was no visible significant increase in particle formation after heat processing of these samples; this corresponded well to particle size distributions shown in Fig. 7.5. The size and number of particles visibly increased after UHT processing when the amount of RSPH was increase beyond 1% in the samples (Fig 7.6). These images can be related to particle size distribution results as discussed above. The optical microscopy images for 8-RSPH were not available due to the very small size of soy peptides that were beyond the measurement capabilities of the instrument used for this analysis.

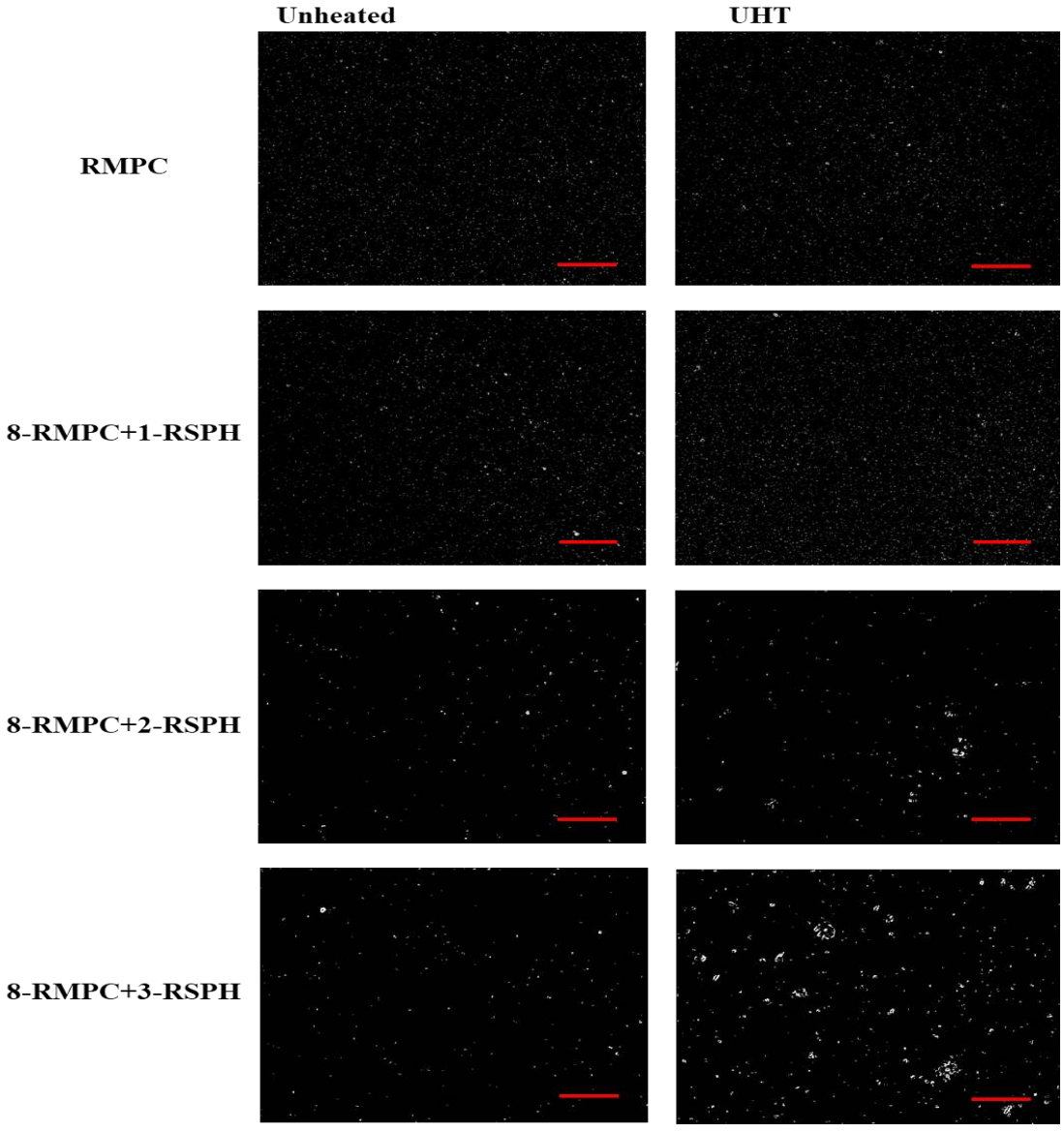

Figure 7.6: Microscopic images of unheated and UHT processed milk protein dispersion. The red scale bar represents a length of 20 µm.

7.3.7 Rheological analysis of protein dispersions

The rheological data showed that the unheated and UHT processed 8-RSPH had significantly lower viscosity than 8-RMPC (Fig 7.7A). The low viscosity of RSPH can be attributed to its high degree of hydrolysis (Wu, Hettiarachchy, & Qi, 1998). A significant increase in viscosity of all three RSPH added 8-RMPC samples was observed as compared to 8-RMPC which could be due to the formation of a small number of protein and peptide complexes formed in pH range 5 to 7 due to electrostatic interactions as explained by Kosters et al. (2013). These complexes could be the small submicron second peak observed in the particle size distribution curve of RSPH added 8-RMPC samples in Fig 7.6A, whereas 8-RMPC had monomodal size distribution curve.

The viscosity of UHT processed protein dispersions with higher RSPH content (8-RMPC+2-RSPH and 8-RMPC+3-RSPH) was significantly higher as compared to 8-RMPC+1-RSPH. Addition of 1-RSPH did not significantly affect the viscosity of 8-RMPC after UHT processing. The viscosity results corresponded well to the UHT behaviour, particle size distribution data and the microscopic observations.

Further assessment of the flow behaviour of UHT processed protein dispersions revealed that they showed a shear thinning behaviour which increased with an increase in their RSPH content. Similar to viscosity data, 8-RMPC+3-RSPH showed highly shear thinning behaviour with its viscosity remaining higher across the shear rate range studied (Fig 7.7B). The flow behaviour index (n) tended to decrease significantly with an increase in RSPH content in the protein mix (Fig 7.7C). This indicated that formation of larger protein aggregates at UHT conditions due to the presence of increased amounts of soy peptides changed the flow behaviour of protein dispersion towards more non-Newtonian type (Beliciu & Moraru, 2011).

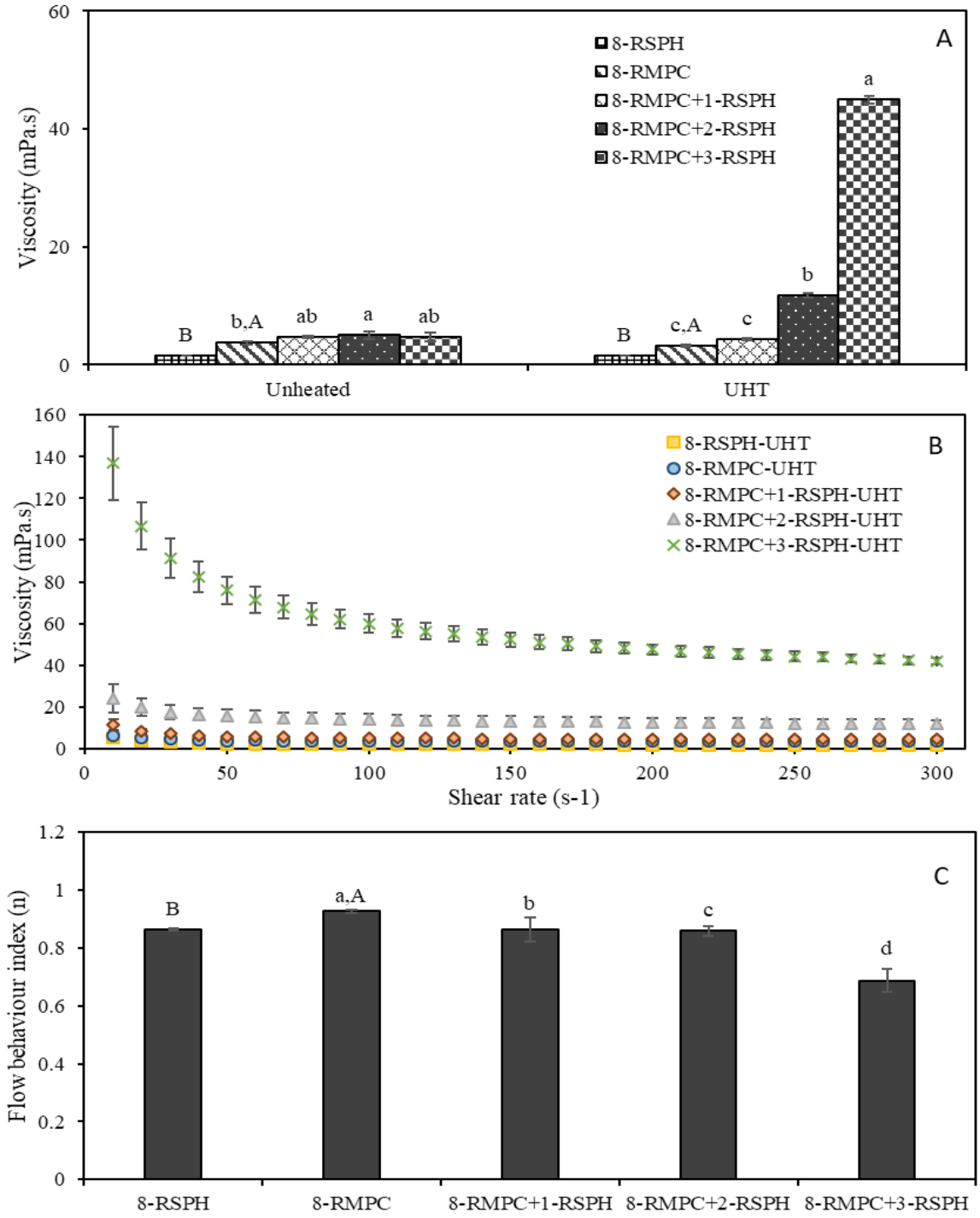

Figure 7.7: Rheological properties of protein dispersions (A) Apparent viscosity at 300 s^{-1} shear rate, unheated vs UHT processed (B) viscosity of UHT treated samples as a function of shear rate (C) Flow behaviour index (n) of UHT processed milk protein dispersions. Error bars represent standard deviation, n=4. Means in a single column with different superscripts are significantly different (P<0.05), capital letters superscripts compare 8-RSPH with 8-RMPC only.

7.3.8 Colour measurements of protein dispersions

Colour of a food product is an important sensory parameter affecting consumer acceptance. Mixing different ingredients for product formulation and processing can result in a change in the colour of the final product (Cheng, Barbano, & Drake, 2019). Comparison of Hunter L, a and b colour values of unheated and UHT processed samples showed that 8-RSPH was significantly darker, more red and less blue than the 8-RMPC (Table 7.4). The addition of different levels of RSPH to 8-RMPC caused a noticeable difference in the colour profile of the resulting protein dispersions even before heating. The lightness of all the RSPH added 8-RMPC samples was significantly lower than 8-RMPC. Additionally, the greenness of the mixed samples also significantly decreased, whereas yellowness increased significantly. Similar results were reported by Friedeck et al. (2003) for a soy protein fortified low fat dairy based ice cream product. An increase in lightness and yellowness and decrease in the greenness of all the samples was observed after UHT processing. This effect of UHT processing on the colour of protein dispersions can be related to non-enzymatic browning caused by Maillard reaction at high temperatures, as also shown by Schamberger and Labuza (2007).

Table 7.4: Hunter L, a and b values of cheese obtained by colorimeter of unheated and UHT processed milk protein dispersions.

Sample	Unheated			UHT		
	L	a	b	L	a	b
8-RSPH	18.75 ± 0.08^{B}	2.76 ± 0.06^{A}	-0.53 ± 0.11^{A}	19.425 ± 0.29^{B}	3.3 ± 0.19^{A}	0.05 ± 0.01^{B}
8-RMPC	67.8 ± 2.76^{aA}	-3.84 ± 0.44^{aB}	-1.40 ± 0.1^{dB}	69.64 ± 2.28^{aA}	$-3.91\pm0.08b^{B}$	0.58 ± 0.04^{cA}
8-RMPC+1-RSPH	61.38 ± 0.91^{ab}	-3.36 ± 0.16^{a}	1.18 ± 0.0^{c}	65.2 ± 1.85^{a}	-2.17 ± 0.86^{ab}	4.21 ± 0.3^{b}
8-RMPC+2-RSPH	60.88 ± 0.57^{b}	-3.29 ± 0.16^{a}	2.41 ± 0.13^{b}	67.46 ± 1.36^{a}	-2.17 ± 0.54^{ab}	6.15 ± 0.07^{a}
8-RMPC+3-RSPH	62.5 ± 1.47^{ab}	-2.94 ± 0.37^{a}	3.76 ± 0.16^{a}	63.45 ± 1.32^{a}	-1.42 ± 0.28^{a}	6.77 ± 0.6^{a}

All results are expressed as the mean ± standard deviation (n = 4). Means in a single column with different superscripts are significantly different ($P<0.05$), capital letters superscripts compare 8-RSPH with 8-RMPC.

7.4 Conclusions

It was observed that RSPH up to 2% protein can be added to 8-RMPC and successfully UHT processed in the experimental conditions studied here. As shown by particle size distribution results and rheological data, a further increase in RSPH content can lead to the larger aggregate formation

and higher viscosity causing excessive fouling in the UHT plant. It was noticed that the presence of higher amounts of soy peptides can induce aggregation of milk proteins by protein-peptide interactions during thermal processing. It can be concluded that RMPC with small, but dietary significant, amounts of RSPH can be successfully UHT processed. This knowledge can be helpful in the formulation of UHT stable dairy-based high protein dispersions with added soy proteins for various food product applications.

CHAPTER 8: GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

8.1 General conclusion

High milk protein beverages possess excellent physiological properties and have significant commercial value. Milk proteins, particularly the whey proteins are used in the formulation of high protein beverages and milk protein system are not very stable at high temperatures. Hence, there is a need to gain a better understanding of the behaviour of concentrated milk protein dispersions during UHT treatment of these products. This research was mainly focused on the effect of type of milk protein ingredients, milk protein content and, other additives and stabilisers on UHT stability and fouling behaviour of high protein beverages. The major outcomes of this project are described below.

Careful selection of milk protein ingredients is required to formulate a UHT stable high protein beverage. Therefore initial part of this PhD project was focused on studying the effect of type of milk protein ingredient and protein concentration on UHT stability of high protein beverages (Chapter 3). Our first study showed that MPC is highly UHT stable at very high protein concentrations; in fact RMPC was found to be UHT stable when it had double the amount of protein as compared to RSMP (14 and 7.5% protein, respectively), although the ionic calcium activity and viscosity of the former was higher than the latter. The lower UHT stability of RSMP were found to be caused by larger protein aggregate formation and destabilisation of casein micelles in 7.5-RSMP at UHT temperatures. High UHT stability of milk protein dispersions made from high protein milk powder, such as MPC85, can be due to the ultrafiltration processing used during their manufacturing, which causes them to have a modified mineral composition as compared to SMP.

The UHT instability of mineral readjusted MPC85 (Chapter 3) even at 7.5% protein concentration suggested that the total mineral composition is responsible for fouling of high protein SMP suspension. The effect of changes in mineral composition on UHT behaviour of MPC powders at different protein concentrations was studied next (Chapter 4). The results showed that compared to SMP lower amounts of minerals, such as calcium, magnesium and potassium in MPC were the major reason behind its high UHT stability. Increasing the calcium, magnesium and potassium concentrations to levels similar in RSMP reduced heat stability of RMPC at 7.5% protein, while the addition of sodium had no significant effect on UHT stability of RMPC. This study further confirmed that membrane filtration process used during MPC manufacturing reduces the protein to mineral ratio, which resulted in high heat stability of milk protein dispersions prepared from it. When the mineral composition was restored to the same protein to mineral ratio as found in SMP, the UHT stability of

RMPC becomes similar to that of RSMP. These results can also be useful for the formulation of mineral fortified high protein beverages using RMPC.

It was concluded that MPC can be used as a base for the development of a UHT stable product. WPC is another popular high protein ingredient, however it showed very low UHT stability. There are reports of caseins' ability to regulate and minimize whey protein denaturation and aggregation. Similar to those studies our work also showed that high concentrations of whey proteins (=5% w/w) can be successfully UHT processed by the inclusion of caseins in the milk protein dispersions (Chapter 5). However, increasing the amount of whey proteins in mix further was not possible due to high level of fouling induced in samples containing 6% (w/w) whey proteins. This suggested that the protective effect of caseins towards whey protein heat stability is strongly dependent on casein to whey protein ratio present in the beverage mix.

In the next part of this PhD project a chocolate flavoured high protein RTD beverage was formulated and UHT processed based on the findings of the first three chapters (Chapter 6). The UHT fouling behaviour of these beverages demonstrated that in order to prepare UHT stable product, κ-carrageenan requirement was influenced by their protein composition. Presence of higher amounts of whey proteins in C:W-Choco lowered their minimum threshold of κ-carrageenan as compared to RMPC-Choco due to entrapment of cocoa particles by whey protein aggregates along with milk-protein κ-carrageenan network. The sensory attributes of these two types of chocolate flavoured high protein beverages were also influenced by their protein composition. The physical properties measured could be related to the sensory attributes perception by an untrained sensory panel. For example, higher viscosity of C:W-Choco-0.01 as compared to RMPC-Choco samples was also perceived by sensory panellists as higher thickness of this sample. The results from this study can help in the formulation and development of a commercial chocolate flavoured high protein beverage.

The next study was an attempt to incorporate plant protein sources into high milk protein beverages (Chapter 7). UHT stability of high protein milk dispersions containing soy protein hydrolysates (RSPH) showed that RSPH up to 2% protein can be added to 8-RMPC and successfully UHT processed in the experimental conditions studied here. As shown by particle size distribution results and rheological data, a further increase in RSPH content can lead to the larger aggregate formation and higher viscosity causing excessive fouling in the UHT plant. It was noticed that the presence of higher amounts of soy peptides can induce aggregation of milk proteins by protein-peptide interactions during thermal processing. It can be concluded that RMPC with small, but dietary significant, amounts of RSPH can be successfully UHT processed. This knowledge can be helpful in

the formulation of UHT stable dairy-based high protein dispersions with added soy proteins for various food product applications.

8.2 Future recommendation

i. The type of milk protein ingredients used for the preparation of a milk protein dispersion greatly influence its UHT fouling behavior. Therefore, it is recommended that UHT fouling behavior of milk protein dispersions prepared from other MPC and WPC powders with different milk protein content to total solids ratio (MPC70, MPC85, WPC30 and WPC60 etc.) can be studied. This will also help in further understanding the role of milk minerals on the heat stability of milk proteins during UHT processing as the ratio of milk proteins to minerals is different in these powders.

ii. The changes in ionic calcium and pH can be recorded before and after the UHT processing to investigate the effect of UHT treatment on these parameters and to study the correlation of these two parameters to product's UHT stability. Additionally measurement of pH and ionic calcium values at processing temperatures (preheating and UHT temperature) can provide more insights into changes occurring to milk components at these temperatures.

iii. We know that greater than 74% ethanol stability is recommended for milk to be suitable for UHT processing. The dependence of UHT stability of various milk protein ingredient based products on their ethanol stability can be studied.

iv. Studying the composition of fouling deposits can also provide further details about the fouling behavior of high protein beverages

v. The mineral salts added high protein beverages showed changes in their UHT fouling behavior. This work can be extended to develop mineral fortified (calcium, iron etc.) high protein drinks to help finding suitable mineral salts for mineral fortification without adversely affecting the UHT stability of the product.

vi. The UHT fouling behavior of chocolate flavored high protein beverages showed that κ-carrageenan improved its UHT stability. Similarly, the effect of κ-carrageenan and some other commercially used stabilizers (sodium alginate, guar gum etc.) on UHT fouling behavior of high milk proteins can be studied in order to minimize fouling.

vii. UHT processing of soy protein hydolysate and milk protein based dispersions showed promising results on the inclusion of plant proteins into dairy based high protein beverages.

Further work on UHT stability of milk protein-plant protein based beverage formulations can be done.

viii. The findings from this work can be scaled up and studied during industrial processing of high protein beverages in order to optimize UHT plant processing conditions for maximizing plant run-time between cleaning in place downtimes.

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Appendices

Appendix 1: Ethics approval letter issued by The University of Queensland

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Sensory Evaluation of Food Product (Dairy, Plant based, Cereals, Meat, Fruits and Vegetables, Confectioneries and Beverages)
Chief Investigator:	Dr Sangeeta Prakash
Supervisor:	None
Co-Investigator(s):	None
School(s):	School of Agricultural and Food Sciences, The University of Queensland
Approval Number:	2019001010
Granting Agency/Degree:	None
Duration:	30 June 2024

Comments/Conditions:

- HREA Form, 02/05/2019
- Consent Form Consumer Panels, 02/05/2019
- Consent Form Trained Panels, 02/05/2019
- Flyer for recruitment of Consumer Panel, 02/05/2019
- Flyer for recruitment of Trained Panel, 02/05/2019
- Intensity Ranking _ Sensory Questionnaire, 03/06/2019
- Paired Preference _ Sensory Questionnaire, 03/06/2019
- Participation Information Sheet Consumer Panels, 02/05/2019
- Participation Information Sheet Trained Panel, 03/06/2019
- Pre-Screening Questionnaire Trained & Consumer Panel, 02/05/2019
- Rating Test _ Sensory Questionnaire, 02/05/2019
- Project Description, 03/06/2019
- Triangle Test _Sensory Questionnaire, 03/06/2019

Note: If this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Sub-Committee:
University of Queensland Science, Low & Negligible Risk Ethics Sub-Committee

This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Sub-Committee representative:
Dr Karen McNamara
Chairperson
University of Queensland Science, Low & Negligible Risk Ethics Sub-Committee

Signature _____ Date 04/06/2019