Milk Protein Concentrates (MPC) with Enhanced Functionalities Produced using Membrane Ultrafiltration

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Doctor of Philosophy

By

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捧上我满满的感激

献给我最亲爱的父亲母亲

你们无私的爱和支持

还有无私的牺牲

化作力量

使我坚强

梦想终成

虽然你们已经离去

你们的爱和精神

永存我心

Abstract

Milk protein concentrate (MPC) has become an important raw material for many applications, including processed cheese, ice cream, yoghurt, fermented dairy and meal replacement beverages. Various processing conditions during MPC production affect their physical functionalities. Quantitative knowledge about the relationship between production conditions and functional properties is limited, especially for the production of MPC using membrane technology.

Understanding this relationship will lead to the establishment of design rules of manufacturing practices for production of MPC with enhanced functionalities. The purpose of this study is to understand how temperature, pH and calcium (Ca) chelators affect the ultra-filtration (UF) process and the functionalities of the MPC. Three major experiments were conducted: i) effect of operation temperature (i.e. 15, 30 and 50 ˚C), ii) effect of the pH of skim milk (i.e. pH 6.7, 6.3, 5.9 and 5.5), and iii) the effect of Ca chelators (EDTA and citrate, at levels of 10, 20 and 30 mM) on the UF operation and the functionalities of the MPC obtained. Raw milk was first used to investigate the effect of temperature, pH and Ca chelators, individually or in combination, on milk at its native state, avoiding the possibilities of the milk protein and minerals being disrupted by any industrial process, then commercial skim milk was selected and used for MPC production and functionality tests.

The parameters tested include casein micelle size and zeta potential, protein profile, Ca content before and after UF processing, the functionalities (solubility, heat stability, emulsion capacity, emulsion activity and emulsion stability) of the MPC (~73 % protein), and membrane performance (permeate flux and membrane fouling).

The decrease in temperature from 50 to 15 °C for the raw skim milk led to an increase in soluble and ionic Ca, and an increase in zeta potential. The effect of temperature on casein micelle size was pH dependant. At pH 6.7 and 6.3, casein micelle size was not affected significantly by the temperature, while at pH 5.9 and 5.5, the casein micelle size decreased with the decrease in

temperature. Changing the pH of raw skim milk from 6.7 to 5.5 caused an increase in soluble and ionic Ca, and an increase in zeta potential. The effect of pH on casein micelle size was temperature dependant. At 15 °C, the casein micelle size decreased with the decrease in pH, while at 30 or 50 °C, the casein micelle size increased with the decrease in pH. Addition of EDTA caused a decrease in the pH, whereas, the addition of citrate caused an increase in the pH. Addition of EDTA caused a decrease in soluble and ionic Ca and an increase in casein micelle size over all the temperatures (15, 30 and 50 °C) and pH ranges (pH 6.7, 6.3, 5.9 and 5.5) tested. Addition of citrate caused a reduction in ionic Ca and an increase in casein micelle size, but the trend in soluble Ca was not consistent. There is no marked effect of EDTA or citrate addition on the zeta potential. UF at 15 °C produced MPC with smaller casein micelle size, better emulsion capability and less membrane fouling in comparison to those produced by UF at higher temperatures (30 or 50 °C).

Lowering the pH of skim milk from 6.7 to 5.5 prior to UF reduced the size of casein micelle in the retentate and resulted in the final MPC with enhanced solubility, heat stability, emulsion capability and emulsion stability. However, lowering the pH of the skim milk prior to UF, on the other hand, reduced the membrane performance with increased membrane fouling and took longer time to achieve similar concentration. The optimum pH was found to be 5.9 among the pH values tested in this study.

Pre-treatment of skim milk feed with Ca chelators (EDTA or citrate) and increase in chelator level from 10 to 30 mM prior to UF reduced casein micelle size, resulted in the MPC with enhanced solubility, heat stability and emulsion capacity, but also reduced membrane performance, increased membrane fouling and took longer time to complete a five times concentration. The enhanced functionality is attributed to the reduced casein micelle size resulted from the partitioning of Ca between retentate and permeate.

This work has provided new insights into the relationship between temperature, pH and Ca chelators on the Ca content, casein micelle size in the concentrates of skim milk, the physicochemical properties of MPC produced, and demonstrated the potential of using temperature, pH and EDTA / citrate to manipulate UF process to produce MPC with enhanced functionalities.

Certificate

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This is to certify that the thesis entitled "**Milk Protein Concentrates (MPC) with Enhanced Functionalities Produced using Membrane Ultrafiltration**" submitted by XiaoXia Luo in partial fulfilment of the requirement for the award of the Doctor of Philosophy in Food Science & Technology at Victoria University is a record of bona fide research work carried out by her under my personal guidance and supervision, and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Dr Todor Vasiljevic

Date:

Student Declaration

I, XiaoXia Luo, declare that the PhD thesis entitled Milk Protein Concentrates (MPC) with Enhanced Functionalities Produced using Membrane Ultrafiltration is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

XiaoXia (Lorna) Luo

Signature: Date: 09/12/2015

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Abbreviations and Terms

A	Activity
Abs	Spectrophotometer Value of Absorbance
AAS	Atomic Absorption Spectrophotometer
ANOVA	One Way Analysis of Variance
AOAC	Association of Official Analytical Chemists
ATR-FTIR	Attenuated Total Reflectance−Fourier Transform Infrared
BSA	Bovine Serum Albumin
Ca	Calcium, one of major mineral in the milk
CF	Concentration Factor
CCP	Colloidal Calcium Phosphate
CCS	Ca Chelating Salt
CM	Casein Micelle
CN	Caseins
DF	Diafiltration
D_h	Hydrodynamic Diameter
DLS	Dynamic Light Scattering
EAI	Emulsion Activity Index
EC	Emulsion Capacity
EDTA	Ethylenediaminetetraacetic Acid
ESI	Emulsion Stability Index
HPLC	High Performance Liquid Chromatography
ICP	Inductively Coupled Plasma
IDF	International Dairy Federation

KN	Kjeldahl Nitrogen
MPC	Milk Protein Concentrate
MF	Microfiltration
MPI	Milk Protein Isolate
NaCN	Sodium Caseinate
NCN	Non-casein Nitrogen
NF	Nanofiltration
NPN	Non-protein Nitrogen
PAGE	Polyacrylamide Gel Electrophoresis
PES	Polyethersulfone
RO	Reverse Osmosis
SAS	Advanced Analytics
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SMP	Skim Milk Powder
TCA	Trichloroacetic Acid
TN	Total Nitrogen
TS	Total Solid
UF	Ultrafiltration
UHT	Ultra High Temperature Processing
VCF	Volume Concentration Factor
WMP	Whole Milk Powder
WPC	Whey Protein Concentration
WPI	Whey Protein Isolate
WPs	Whey Proteins

β-Lg	β-Lactoglobulin	
α-La	α-Lactoalbamin	
Ig	Immunoglobulins	
ϕ	Oil Volume Fraction in the Emulsion	
λ	Wavelength	
ΔT	Turbidity Drop	
Δt	Time Interval or Change	
L	Path Length of Cuvette	
A	Membrane effective surface area	m^2
J	Permeate flux	$m^3/m^2.s$
t	Permeation time	s
V	Permeate volume	m^3
ΔP	Transmembrane pressure	Pa

List of Publications and Conference Presentations

Refereed Journal Publications

X. Luo, L. Ramchandran, T. Vasiljevic. (2015). Lower Ultrafiltration Temperature Improves Membrane Performance and Emulsifying Properties of Milk Protein Concentrates. *Journal of Dairy Science and Technology*, 95 (1), 15-31.

X. Luo, T. Vasiljevic, L. Ramchandran. (2015). Effect of Adjusted pH Prior to Ultrafiltration of Skim Milk on Physical Functionality of Milk Protein Concentrate. *Journal of Dairy Science,* 99 (2), 1083-1094.

Manuscripts in Preparation

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Chapter 1. **Introduction**

Dairy industry in Australia has developed since the first cows arrived in the 1700's, then underwent rapid growth in the last century to a current gross value of over $4 billion annually, both in size and range, and became the third largest agriculture industry in Australia and the third largest dairy exporter in the world, comprising about 10 % of the global dairy market (Dairy Australia, 2013, 2014).

The demands for a milk product that could solve the economic problems of fresh milk regarding storage and transport requirements resulted in the development of milk powders including milk protein concentrate (MPC) powders.

In the milk, ~87 % is water; ~4.6 % is lactose, ~3.9 % is fat, ~3.3 % is proteins (whey protein and casein) and < 1 % is minerals and others (Walstra & Jenness, 1984). The water can be removed by different drying processes such as freeze drying or spray drying (Henning, et al., 2006). In order to increase the protein content in powders, removal of lactose and minerals before drying is desirable resulting in MPC powders having high protein content (Le Graet & Brulé, 1982; McKenna, 2000). Membrane ultrafiltration (UF) is one of the technologies widely used in dairy industry to remove lactose and minerals, which have smaller molecular sizes than that of proteins (whey protein and casein) (TetraPak, 1995).

MPC is skim milk that has been concentrated by UF to retain most of the proteins (casein and whey protein) while removing much of the water and some of the lactose, ash and other solids. MPC contains casein and whey protein in nearly the same proportion, as they are naturally found in skim milk. MPC is ideal for a large range of applications, including cheese products, cultured products, dairy−based beverages, paediatric nutrition, medical nutrition, weight management products, powdered dietary supplements for sports food and clinical nutrition products. MPC is used as a source of dairy protein in prepared foods because of its bland flavour that allows other flavours to develop fully (Oldfield & Singh, 2005).

Demand for MPC has grown significantly in recent years. The worldwide market for MPC as an ingredient and consumer products formulated using MPC has grown over the last decade. For example, the production of MPC worldwide has increased from 40,000 metric tons in 2000 to approximately 270,000 metric tons in 2012. One major reason for increased MPC production and demand is that more products have been launched using MPC as a food ingredient (Dairy Australia Annual Report, 2013).

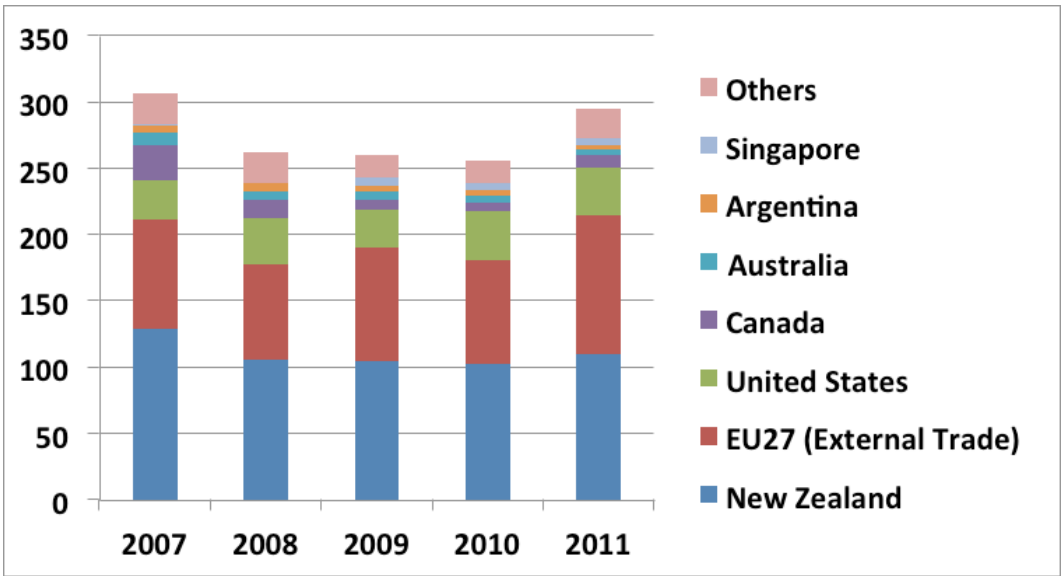


Figure 1.1. The major exporters of MPC and related products (in 1,000 mt) (Dairy Australia Annual Report, 2013).

New Zealand is the world's leading MPC producing country, followed by the EU (Figure 1.1). They are also the largest producers and exporters of casein. World production and exports of MPC are dominated by New Zealand, the EU and United States. The US production is increasing rapidly and is the world's largest market for MPC. The Asia, in particularly the China rapidly becomes large consumer of MPC recently. Australia is third producer and exporter of milk powder but produces an array of different milk powders, more focused on whole milk powder (WMP) and skim milk powder (SMP). (Dairy Australia Annual Report, 2013).

The major problem faced by the manufacturers of MPC powders is related to the functionality of MPC, such as low solubility and emulsifying capacity, which limits the food applications of MPC. Most of the studies conducted in this area focused on reconstituted MPC powders. Very little work has been carried out to understand the effect of processing conditions, related to the functional properties, especially for MPC produced using membrane systems.

Various processing conditions during manufacture of MPC can affect their functionalities. In terms of functionalities, solubility is the most important functional property for proteins in liquid form. Solubility is essential for other functionalities since insoluble proteins cannot perform any useful activities. Emulsifying property is also an important functionality for MPC application (Cameron, et al., 1991). The solubility, heat stability and, in particular, the emulsion property (emulsion capacity and stability) are related to the protein particle size (casein micelles) (Ye, 2011). Casein micelle size during MPC production can be affected by the milk environment, such as temperature, pH and Ca concentration during the UF processing, however, the exact understanding of the effect of such parameters on the casein micelles and its relation to the functionality of MPC powders is lacking. Fundamental research on the functional properties of proteins and the change of these properties induced by process are essential to improve MPC functionality. Understanding such relationship will enable the industry to manipulate processing parameters (e.g. operation parameters, pH and chelators) to achieve the production of MPC with enhanced functionalities.

There exist inconsistent results from previous research regarding the alterations to casein micelles during membrane processing. The size of casein micelles was reported to be unchanged (Karlsson, et al., 2007), increased (Singh, 2007; Srilaorkul, et al., 1991) or decreased (Erdem & Yuksel, 2006) as a result of UF. Martin, et al. (2010) compared the functionality of spray dried MPC powder with skim milk powder and indicated that there was no major change in the micelle size during UF / Diafiltration (DF) or evaporation. This is in contrast to the manufacture of skim milk powder (SMP), in which micelle size has been found to increase during evaporation (Martin, et al., 2007). Presumably either the removal of minerals during UF / DF or the lesser extent of concentration in the

evaporator prevents a similar increase in micelle size from occurring during MPC manufacture. Possible factors that affect micelle size during MPC manufacture could be the changes to the pH, calcium and phosphate equilibrium, milk concentration or possible whey protein denaturation experienced during conventional spray drying process, but the exact reason remains unclear. Therefore an investigation on the effect of process and membrane processing parameters of MPC making, such as temperature, pH and changing Ca concentration, on the casein micelles is necessary.

Additional research also found that the functionalities of MPC decreased with an increasing proportion of calcium in the mineral fraction of the product (Ye, 2011). Therefore change the casein micelle size, and / or concentration of Ca content in the milk can contribute to the improvement of MPC functionalities. Whereas the micelles size, the fraction of milk Ca can be optimised by the control of the processing conditions. This project was designed to address such gaps.

The aim of this study was to acquire a thorough understanding of the fundamental mechanisms underpinning the membrane processing of milk streams for production of MPC with required functionality. This will lead to a development of an improved membrane processing system that would produce MPC with enhanced functionality, especially the emulsifying capacity and required solubility.

The specific objectives of this study were:

- To gain fundamental understanding on how processing conditions (pH, temperature, membrane) affect casein micelle behaviour during membrane processing.
- To relate processing conditions (pH, temperature, chelating agent) to MPC functionality.

Following this chapter, chapter 2 of this thesis presents a review on relevant literature regarding the milk proteins (whey and casein) and casein micelles, milk salts especially Ca and its equilibration in the milk, and the influence of processing parameters (temperature, pH, and addition of chelating agents) and UF processing on the MPC production.

Chapter 3 describes the experimental designs, techniques, materials and methods used in this study.

Chapter 4 describes the investigation on the effect of temperature, pH and the addition of chelating agent on the casein micelle size, zeta potential, structural characteristics of proteins, the fractionation of Ca (soluble and free ion) and their influence on the physicochemical properties of raw milk.

Chapter 5 describes the investigation of the effect of temperature (15, 30 and 50 °C) of milk for MPC production using UF process and the functionalities (solubility, heat stability and emulsion properties) of MPC.

Chapter 6 describes the investigation of the effect of pH (pH 6.7, 6.3, 5.9 and 5.5) of skim milk on the MPC processing and MPC functionalities.

Chapter 7 describes the investigation of the effect of addition of Ca chelating agent (EDTA and sodium citrate at concentration of 10, 20 and 30 mM) on milk (pH 5.9) UF processing and MPC functionalities.

Chapter 8 provides summary and general conclusions of this project and provides some recommendations for future work.

All the referenced articles in this thesis are listed at the end of this thesis in Chapter 9.

Chapter 2. **Literature Review**

2.1 Milk Composition and Its Biological Role

Milk is a white or yellowish white complex fluid containing several hundred molecular species. The principal constituents are water, lipids, sugar (lactose) and proteins that exist in various forms such as fat globules as a discontinuous emulsion phase and casein micelles in colloidal suspension within the continuous aqueous phase that contains all the other constituents (Holt, 1997; Fox, 2003). The principal components, protein (3.3 %), lactose (4.6 %), fat (3.9 %) and ash (0.78 % includes milk minerals) are the solutes while water is the solvent present in milk. Milk also contains organic acids such as citrate, and a range of vitamins that do not fit conveniently into the four major categories. Typical composition of milk is summarised in Table 2.1 (Walstra & Jenness, 1984).

Table 2.1. Composition of bovine milk (adapted from Walstra & Jenness, 1984)

Component	Average Content (Wt %)	Range (Wt %)
Water	87.3	85.5−88.7
Fat	3.9	2.4−5.5
Protein	3.25	2.3−4.40
Casein	2.6	1.7−3.50
Whey protein	0.65	0.4−0.65
Lactose	4.6	3.8−5.30
Mineral substance	0.7	0.53−0.80
Organic acid	0.18	0.13−0.22
Miscellaneous	0.14	0.10−0.19

Values are mean values of a range of different components of milk in different cows.

The physical properties of milk are similar to water but are modified due to the

presence of various solutes in the continuous phase and by the degree of dispersion of the emulsified and colloidal components (Fox & McSweeney, 1998).

In much of the world, particularly in the Western world, bovine milk commonly called cow milk, accounts for nearly all the milk processed for human consumption (Swaisgood, 1996). In this thesis all investigation will focus on cow milk and its proteins.

In many European countries, the USA, Canada, Australia and New Zealand, about 30 % of dietary protein is supplied by milk and dairy products. As a dietary item, milk has many attractive features:

- Nutritionally, it is the most complete single food available
- It is free from toxins and anti-nutritional factors
- It has a pleasant and attractive flavour and mouthfeel.

2.2 Milk Proteins

Research on milk proteins dated from 1814, when the first paper on the subject was published by Berzelius (1814). Later in 1938, the general complexity and heterogeneity of the milk protein system was described and milk proteins were understood to be composed of caseins, lactalbumin, lactoglobulin, protease peptone and non-protein nitrogen which represents approximately 78 %, 12 %, 5 %, 2 % and 3 %, respectively, of the nitrogen in bovine milk (Rowland, 1938). The current status of knowledge on milk proteins was described by McSweeney & Fox (2013).

Milk protein as one of important composites plays a critical role in the milk system. Milk contains 30 - 36 g of protein / L of milk and it rates highly in nutritional quality (Swaisgood, 1996). The properties of milk and most dairy products are affected more by the proteins they contain than by any other constituent. The milk proteins have many unique functional properties, such as emulsifying properties, foaming properties and thickening properties, etc. Because of these functional properties, they have significant technological importance, and they have been studied extensively and are probably the best

characterised food protein system (McSweeney & Fox, 2013).

Milk proteins are classified as either caseins or whey proteins. When milk is acidified to pH 4.6 (isoelectric point), about 80 % of total bovine milk protein precipitates out of suspension. This fraction is collectively termed casein (Walstra & Jenness, 1984). The liquid remaining after isoelectric precipitation of casein from skimmed or whole milk is called whey. It is a dilute solution of proteins, referred to as whey or serum proteins, which are present at a concentration of approximately ~ 0.7 % in bovine milk (O'Mahony & Fox, 2013). The major bovine milk proteins and their molecular weight, concentration in the milk and their biological functions are shown in Table 2.2.

Table 2.2. Major bovine milk proteins and some of their biological functions (adapted from Korhonen & Pihlanto, 2007)

Protein	Average concentration in milk (g/L)	Molecular weight (Da)	Major biological Functions
Whey protein			
α-lactalbumin (α−La)	1.2	14200	Lactose synthesis in mammary gland
β-lactoglobulin (β−Lg)	3.3	18400	Retinol carrier, antioxidant and vitamin−A−binding protein
Bovine serum albumins (BSA)	0.3	66300	Peptide source
Immunoglobulins (Ig)	0.5−1.0	150000 1000000	Immune protection
Lactoferrin	0.1	80000	Antimicrobial, antioxidant and anti−inflammatory
Caseins	28	19000-25000	Mineral carrier and peptide source
α_{s1}−casein	10	23612	−
α_{s2}−casein	2.6	25228	−
β−casein	9.3	23980	−
κ−casein	3.3	19005	−

Milk proteins are of significant physiological importance to the body for functions relating to the uptake of nutrients and vitamins and they are a source of biologically active peptides. Whey protein is a complete protein whose biologically active components provide additional benefits to enhance human health. Whey protein contains an ample supply of the amino acid cysteine. Cysteine appears to enhance glutathione levels, which has been shown to have strong antioxidant properties that can assist the body in combating various diseases (Bounous, 2000). In addition, whey protein contains a number of other proteins that positively affect immune function (Ha & Zemel, 2003). Whey protein also contains a high concentration of branched chain amino acids that are important for their role in the maintenance of tissue and prevention of catabolic actions during exercise (MacLean, et al., 1994).

Table 2.3. Some structural and chemical characteristics of milk proteins (Kinsella, et al., 1989)

Protein Conformation	Whey			Casein			
	Globular			Extended			
	β−Lg	α−La	BSA	α_{s1}−	α_{s2}−	β−	κ−
Total No. of residues	162	123	581	999	207	209	169
Apolar residues [%]	34.6	36	28	36	40	33	33
Proline residues	8	2	28	17	10	35	20
Phosphoryl group	15	12	59	14	24	11	9
Disulphide bonds	0	0	0	8−9	9−12	5	1
Thiol group	2	4	17	0	1	0	1
Isoionic point	5.2	4.3	5.3	4.96	5.27	5.2	5.54
Secondary structure (%)							
α−helix	15	26	54	−	−	9	23
β−sheet	50	14	18	−	−	25	31
β−turns	18	−	20	−	−	−	24
Unordered	−	60	−	−	−	66	−

Similar to whey, casein is a complete protein and also contains the minerals of Ca and phosphorous. Casein is the major component of protein found in bovine milk accounting for nearly 80 % of total protein and is responsible for the white

colour of milk (Walstra, et al., 2006). It is the most commonly used milk protein in the industry today (Dairy Australia, 2013; The world casein & caseinates market, 2014; FAO, 2013).

The difference in structure and chemical characteristics between whey proteins and casein is summarised in Table 2.3. The important difference between casein and whey proteins is that caseins are heat insensitive, whilst whey proteins denature at high temperatures (Visser & Jeurnink, 1997). Both the proteins are reviewed separately in the following sections.

2.2.1 WHEY PROTEINS

Milk whey proteins are classified as globular proteins that do not precipitate at pH 4.6 but denatures upon heating to high temperatures (Sawyer, et al., 2002). It can be soluble in saturated NaCl and after rennet-induced coagulation of the caseins. Whey protein is not sedimented by ultracentrifugation, with or without added Ca^{2+} (Creamer & MacGibbon, 1996).

Whey proteins constitute 20 % of the proteins in milk and include β−lactoglobulin (β−Lg: approximately 3.2 g / L), α−lactalbumin (α−La: approximately 1.2 g / L), BSA (approximately 0.4 g / L), and immunoglobulins (approximately 0.7 g / L) (Raikos, 2010). Commercially they are obtained from whey, a by−product of cheese manufacturing. In recent years, research on whey proteins has attracted interest from many scientists because of their biological value, which is the highest among all known proteins, as they can be effectively utilized by humans and provide a considerable amount of essential amino acids for growth (Hambraeus & Lonnerdal, 2003).

Whey proteins are easily denatured (as low as 70 °C) during thermal processing, a process commonly applied to milk during industrial processing in order to ensure the microbial safety of dairy products as well as to extend shelf life (McKinnon, et al., 2009). As a result they undergo conformational changes due to unfolding of the initially folded molecules. The denaturation of whey proteins by heat is a major processing issue in the dairy industry (Al−Attabi, et al., 2009). During heat processing of milk, the formation of whey protein aggregates or complexes leads to fouling of heat exchangers which, in turn,

limits run times of industrial plants, reduces heat transfer, and increases pressure drop (Delplace, et al., 1994; Visser & Jeurnink, 1997). Denaturation of whey proteins can also cause modifications to the chemical and nutritional properties of milk.

Depending on the physicochemical conditions in milk, the denaturation process is either reversible, where partial unfolding of the whey proteins takes place with a loss of helical structure, or irreversible where an aggregation process occurs involving sulfhydryl (–SH) / disulfide (S–S) interchange reactions (Vasbinder, et al., 2003) and other intermolecular interactions, such as hydrophobic and electrostatic interactions (McMahon, et al., 1993; Hoffmann & vanMil, 1997; Anema & Li, 2000). These denaturation and aggregation reactions are of considerable interest to dairy scientists because knowledge of them is essential for devising ways to manipulate the chemical and nutritional properties of dairy products. Of practical interest is the production of heat-stable beverages containing high levels of whey proteins for which whey proteins with improved heat stability is a requirement.

In general, whey protein aggregation involves the interaction of a free –SH group with the S–S bond of cystine-containing proteins such as β−Lg, α−La, and BSA via –SH / S–S interchange reactions (Considine, et al., 2007). This protein−protein interaction leads to irreversible aggregation of proteins into protein complexes of varying molecular size depending on the heating conditions and protein composition. Knowledge of ways of inhibiting the formation of these protein complexes is needed in order to minimize the negative practical consequences that may arise. Over recent decades, studies on the mechanism of formation of these protein complexes have been conducted and various approaches have been followed in order to prevent such protein aggregations (Sawyer, 2013).

2.2.1.1 α−Lactalbumin (α−La)

α−La is a whey protein unique to milk and is present in the milk of all mammalian subdivisions. The structure of α-La consists of 4 α−helices contained in a bundle like form. This general structure can change to three different forms: Ca bound, Ca free, and low pH form, which results from its

ability to bind to Ca and other ions (Creamer & MacGibbon, 1996). The binding of Ca to native α−La is very strong (Kronman, et al., 1981; Schaer, et al., 1985). It has strong effect on molecular stability of milk (Ikeguchi, et al., 1986). In the native protein, Ca binding is necessary for native disulfide bond formation and refolding (Ewbank & Creighton, 1991; Rao & Brew, 1989). For denatured α−La, more than two orders of magnitude of the rate of refolding can be accelerated by Ca while maintaining the general pathway of the transition (Forge, et al., 1999; Kuwajima, et al., 1989). The folding rate of α−La is dependent on the Ca concentration, when Ca concentration is reduced the protein is more susceptible to denaturation (Walstra & Jenness, 1984).

2.2.1.2 β−Lactoglobulin (β−Lg)

β−Lg which represents around half of the whey proteins (Thompson, et al., 2009) has been studied extensively. The structure of β−Lg features an eight−stranded β-barrel and a three turn helix lying parallel to three β−strands (Creamer & MacGibbon, 1996; Visser & Jeurnink, 1997). The amino acid sequence of the entire structure contains five cysteine residues, of which four are bound via disulphide linkages, and the fifth, at room temperature is buried within a group of hydrophobic residues between the helix and the β−strands (Creamer & MacGibbon, 1996; Sawyer, et al., 2002; Visser & Jeurnink, 1997; Walstra & Jenness, 1984). The primary function of β−Lg is not nutritional, as it is very resistant to proteolysis in its native state (Thompson, et al., 2009). The free thiol group plays a significant role in the changes of β−Lg and its interactions with other milk proteins during thermal processing. At elevated temperatures, β−Lg unfolds, exposing the reactive thiol group, resulting in irreversible aggregation with itself and other molecules such as κ−casein and α−La (Visser & Jeurnink, 1997). The structure and aggregation state of β−Lg is strongly dependent on pH (Creamer & MacGibbon, 1996; Sawyer, et al., 2002; Visser & Jeurnink, 1997), and the effect of temperature varies as a function of pH. Below pH 2.0, β−Lg exists as a monomer; when closer to its isoelectric point (pH = 5.1), it exists as an octamer at room temperature. Between pH 5.5 and 6.5, β−Lg exists as a dimer at room temperature. Dissociation to a monomer occurs at temperature above 50 °C. Further heating to 65 °C results

in unfolding of the structure and subsequent exposure of the free SH group, which is followed by aggregation (Creamer & MacGibbon, 1996).

2.2.1.3 Bovine serum albumins (BSA)

BSA is a large protein and it makes up approximately 10 to 15 % of total whey protein in bovine milk. It can irreversibly adsorb onto either hydrophobic or hydrophilic surfaces regardless of the surface charge (Frateur, et al., 2007; Fukuzaki, et al., 1995; Nasir & McGuire, 1998), with the presence of divalent cations in milk (Ca^{2+} & Mg^{2+}) enhancing this adsorption (Zanna, et al., 2006). The physicochemical property of BSA has been studied extensively as an example of a highly structured but flexible protein (Mulvihill & Fox, 1989). While BSA has the ability to form heat−induced intermolecular disulfide bonds with α−La and β−Lg (Havea, 2006) and influences the denaturation, aggregation and gelation properties of β−Lg (Kehoe, et al., 2007), it probably has little effect on the physicochemical properties of milk protein ingredients due to its relatively low concentration.

2.2.1.4 Immunoglobulins (Ig)

Mature bovine milk also contains 0.6 − 1 g immunoglobulin / L of milk. The physiological function of Ig is to provide immunity to the neonate.

But essentially, there are five classes of Ig: IgA, IgG (with subclasses, e.g., IgG occurs as IgG_1 and IgG_2), IgD, IgE and IgM. IgA, IgG and IgM are present in milk (Hurley, 2003). IgG consists of two heavy (large) and two light (small) polypeptide chains linked by disulfides. IgA consists of two such units (i.e. eight chains) linked by secretary component (SC) and a junction component (J), while IgM consists of five linked four-chain units. The heavy and light chains are specific to each type of Ig.

2.2.2 CASEINS

Understanding the casein micelles and the effect of processing condition on the physicochemical properties of casein micelles is fundamental to this study. Therefore a comprehensive review on the casein, casein micelles and casein

micelles during processing as well as Ca distribution and related alterations within the casein micelles will be reviewed in this section. The four major types of casein fractions (α_{s1}−, α_{s2}−, β− and κ−casein) represent approximately 37, 10, 35 and 12 % of the casein in bovine milk system. All four casein fractions are phosphorylated to variable degrees that are characteristic to each form (Holt, 1992; Walstra, 1979).

2.2.2.1 Casein micelles

Caseins together with phosphate, Ca and traces of citrate form structures called casein micelles. Casein micelles are colloidal, poly−dispersed and spherical with an average diameter of 50 − 200 nm (Holt, 1992). The micelles are highly hydrated, binding ~2.0 − 4.0 g H_2O / g protein (depending on how hydration is measured) (De Kruif, 1999; Morris, et al., 2000; Walstra, 1979). They are heterogeneous, dynamic with packing and highly porous structure (Holt, 1997; Walstra, 1979). Casein micelles are composed of four different casein molecules, namely α_{s1}−, α_{s2}−, β−, and ƙ−casein, in a molar ratio of 4: 1: 4: 1 (Gaucheron, 2005; Walstra, 1990; Panouillé, et al., 2005; Phadungath, 2005). These molecules differ in hydrophobicity, net charge, phosphate concentration and Ca sensitivity. They exhibit self−association, depending on the physicochemical conditions (Holt, 1992; Horne, 1998; Panouillé, et al., 2005).

Casein micelles contain about 7 g minerals per 100 g casein, which is called colloidal Ca phosphate (CCP). CCP is more than just amorphous Ca phosphate, as it also contains sodium, potassium, and citrate (Gaucheron, 2005; Lyster, et al., 1984; McGann, et al., 1983; Walstra, et al., 2006). The exact composition of CCP depends on the ionic environment, indicating that it has ion−exchange properties (Gaucheron, 2005; Walstra, et al., 2006). The nano-cluster of CCP have estimated diameter of about 2.5 nm (Walstra, et al., 2006; Holt, 1992; Gaucheron, 2005; McGann, et al., 1983; Marchin, et al., 2007). CCP acts as “glue” in the micelles: the more CCP present in the micelles, the more rigid the micelles will be (Walstra, 1990). A typical casein micelle contains about 10^4 polypeptide chains of casein molecules associated with about 3×10^3 nanoclusters of CCP (Holt, 1992; Gaucheron, 2005). About two thirds of the casein is directly bound to the colloidal Ca phosphate through

the negative charges of the phosphoserine residues, reducing the electrostatic repulsion in the casein micelles (Holt, 1997). Casein micelles in milk have a voluminosity of about 3 − 4 mL water per g of dry casein (Walstra, et al., 2006; Dalgleish, 2010; Korolczuk, 1981; McMahon & Brown, 1984), giving them a spongelike colloidal structure, since they hold more water than dry matter. Relatively little of this water, around 0.5 g H_2O per g dry casein, is directly bound to casein (McMahon & Brown, 1984).

The biological importance of micelle structure relates to the comparative colligative properties of individual casein molecules on one hand and micelles on the other. The main functions of caseins in milk system are to safely transport Ca and phosphate to the mammary gland without precipitation (Creamer & MacGibbon, 1996). These micelles are roughly spherical particles with diameters up to 600 nm as intact casein micelles naturally present in milk, which is the colloidal phase of milk. These particles scatter light and are mainly responsible for the white colour of milk (the small fat globules also scatter light weakly); they can be 'visualized' by the ultramicroscope (essentially a device for measuring light scattering). The milk of all species is white, suggesting that all contain casein micelles. Some casein (such as β−casein) is able to shift between the serum and the colloidal phase, particularly as a result of changes in temperature (Creamer, et al., 1977; Udabage, et al., 2003). Upon cooling, β−casein is liberated from large micelles and κ−casein is solubilised from small micelles (Ono, et al., 1990). At temperatures above 70 °C, micelle size increases due to heat induced protein aggregation and attachment of denatured whey proteins (Anema & Li, 2003a).

The Ca and phosphate within the micelles are known to exchange between the serum and the micelles (McMahon & Brown, 1984). CCP is more stable at high temperature (Morr, 1967a) and removal of CCP will not change the size distribution and protein composition of casein micelles (Griffin, et al., 1988; Lin, et al., 1972). κ−casein, which represents about 15 % of total casein, covers part of the casein micelle surface (Dalgleish, 1998). It stabilizes the Ca sensitive caseins (α_{s1}-, α_{s2}-, β−caseins), which represent about 85 % of total casein (Fox & McSweeney, 1998). Some of the principal properties of casein micelles are summarized in Table 2.4 and individual caseins will be reviewed in this chapter.

Table 2.4. Average characteristics of casein micelles (Fox, 2003).

Characteristic	Value
Diameter	130 − 160 nm
Surface	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g / cm^3
Mass	2.2×10^{-15} g
Water content	63 %
Hydration	3.7 g H_2O/g protein
Voluminosity	4.4 cm^3 / g
Molecular weight (hydrated)	1.3×10^9 Da
Molecular weight (dehydrated)	5×10^4 Da
Number of peptide chains (MW: 30,000 Da)	10^4
Number of particles per mL milk	10^{14} − 10^{16}
Whole surface of particle	5×10^4 cm^2 / mL milk
Mean free distance	240 nm

2.2.2.2 α−Caseins

α−Caseins (α_{s1}-, α_{s2}-) stabilize milk proteins by both refolding properties and chaperone−like activity (Sakono, et al., 2011). The solubility of α−caseins is marginally influenced by temperature but strongly influenced by pH (Post, et al., 2012). α_{s1}−casein, which has two predominantly hydrophobic regions (“A” and “B”) and one highly charged polar zone (“C”) (Fox & McSweeney, 1998), exists as a loose flexible polypeptide chain. α_{s1}−Casein A is soluble at Ca^{2+} concentrations up to 0.4 M below 33 °C, whilst α_{s1}−casein B and C are insoluble and form coarse precipitates when Ca^{2+} is present at concentrations above 4 mM (Singh & Flanagan, 2006). The presence of α_{s1}−casein A modifies the behaviour of α_{s1}−casein B, which is then soluble in 0.4 M Ca^{2+} at low temperature (Singh & Flanagan, 2006). α_{s2}-Casein is more complex than α_{s1}−casein and is generally present as a mixture of four phosphorate forms with 10 − 13 phosphates (Thompson, et al., 2009). It has a dipolar structure where

the N terminus is negatively charged, and the C terminus is positively charged (Fox & McSweeney, 1998). It is insoluble in Ca solution over 4 mM (Singh & Flanagan, 2006).

2.2.2.3 β−Casein

β−Casein has an unordered structure, which has a high degree of segmental motion (Nasir & McGuire, 1998). It has a strong negatively charged N terminus and an uncharged hydrophobic tail (Krisdhasima, et al., 1993). It can stabilise the Ca phosphate in solutions (Holt, 2004) but in a temperature−dependent manner (Singh & Flanagan, 2006). This could be because β−casein undergoes a temperature−dependent conformational change in which the content of poly−proline helix decreases as a function of temperature (Singh & Flanagan, 2006). By following a sequential process, β−casein is able to form micelle−like aggregates (O'Connell & Fox, 2003) and this is primarily attributed to diffusive motion, long range concentration fluctuations (De Kruif & Grinberg, 2002) and hydrophobic interactions (Pierre & Brule, 1981). The experiments related to β−casein micelle formation fit well with the thermodynamics underlying the shell model of casein micelle structure, which has been well discussed by many researchers (Kegeles, 1979; Mikheeva, et al., 2003).

2.2.2.4 κ−Casein

κ−Casein has a flexible structure that includes positively charged hydrophobic regions, negatively charged Polar Regions and connecting domains (Fox & McSweeney 1998). It contains high amount of β−sheet structure which can form close association with phosphorylated caseins (Farrell, et al., 2003). κ−casein is markedly different from the other three caseins in terms of its solubility characteristics. It is insensitive to Ca and is soluble in Ca solutions at all concentrations up to those at which general salting out occurs (Singh & Flanagan, 2006). It is able to stabilize α-casein and β-casein by forming an exterior coating of k-casein, terminate casein aggregation so that no further casein protein adsorption can be achieved, even in the presence of Ca (Liu, et al., 2013). This is consistent with the understanding that it forms the outer layer of casein micelles, helping to stabilize the colloidal particles (Nagy, et al., 2012).

The proportion of κ-casein in casein micelles has been shown to be inversely related to their size (Davies & Law, 1983).

2.2.2.5 Structure of casein micelle

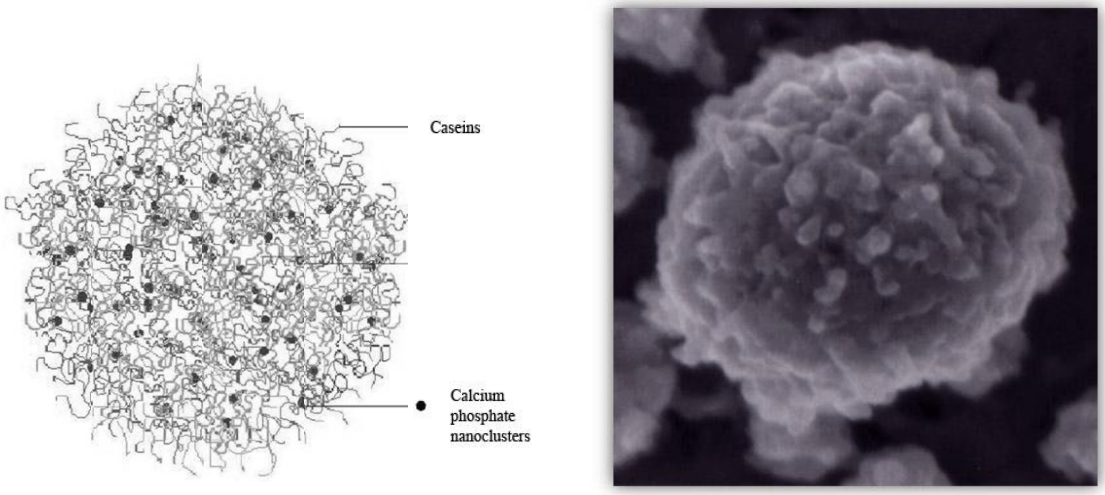
Casein may be present in the form of intact casein micelles (e.g. present in MPC or isolate, ultrafiltered milk and micellar casein isolate) or small casein aggregates (e.g. sodium or Ca caseinate). Intact casein micelles are structurally similar to those naturally present in milk. The structure of casein micelles has been the subject of investigation for years (Walstra & Jenness, 1984; Horne, 2006; Dalgleish & Corredig, 2012). The knowledge of micelle structure is important because the stability and behaviour of the micelles are central to many dairy processing operations.

2.2.2.6 Casein micelle models

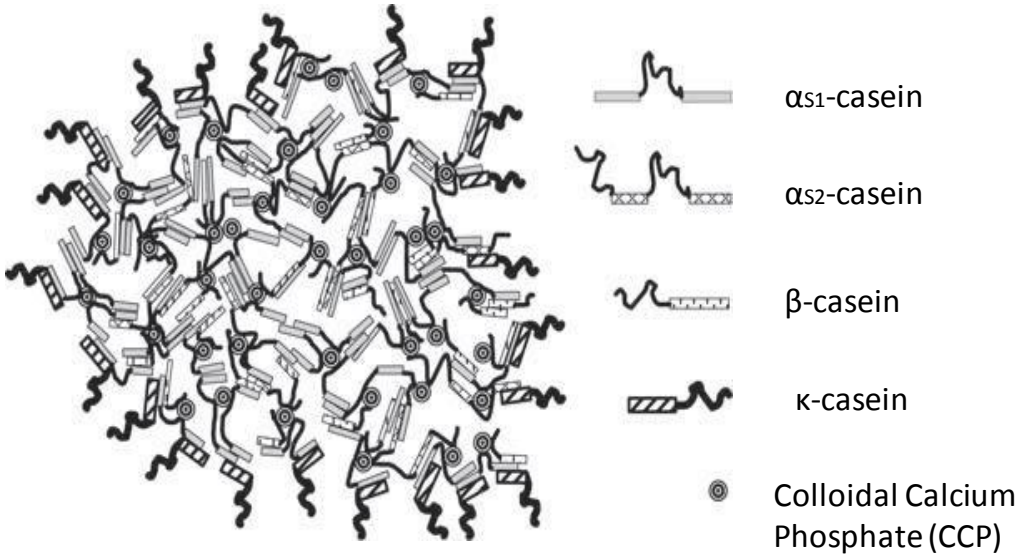
Various models of the casein micelles have been proposed and refined over the past 40 years. The nature and structure of the casein micelles have been extensively studied and the various structural models that were proposed over the years is summarised in Table 2.5.

Table 2.5. Proposed casein micelle structure models.

Model	Presented by	Core principle of casein micellar
Sub−micelles	Waugh (1958)	Build up internal structure with formation of roughly spherical casein micelles and have a hairy outer layer on the surface
Dual−binding	Horne (1998)	Assembly and growth by a polymerization process involving the links between Ca phosphate nanoclusters and through hydrophobic regions of caseins.
Nanocluster	Holt (1992)	A gel−like structure with tangled web of flexible casein and Ca phosphate nanocluster−like “cherry stones”.
Sponge	Bouchoux, et al. (2010)	Soft micro-emulsion−like objects when responding to environment changes.



(a)



(b)

Figure 2.1. Recent models of the casein micelle. (a) The casein micelle (Walstra & Jenness, 1984) and SEM image of a casein micelle (Dalgleish & Corredig, 2012). (b) The dual binding model of the casein micelle from Horne (1998) and the schematic representation of the casein micelle adapted from Horne (2006).

The casein micelles are complex and dynamic entities, each of the models above was developed from different angle and method of simplification, and the exact structure of the casein micelle is still not fully understood. The widely accepted model is based on the concept; core coat, internal structure and subunit or sub micelles (Figure 2.1a, b), a) the casein micelle and SEM image of a casein micelle (Walstra & Jenness, 1984; Dalgleish & Corredig, 2012); and b) the dual−binding model of the casein micelle (Horne, 1998) and the schematic representation of the casein micelle (Horne, 2006), there is no precise description of the micelle internal structure to date. The models further indicate that CCP plays an integral role in the structure of the casein micelle, and the milk salt, in particular the calcium as key component plays an important role within CCP and maintains casein micelles structure.

2.2.3 CASEIN PROPERTIES

2.2.3.1 Solubility

Solubility is the most important functional properties for proteins in fluid and is also essential for other functionalities since insoluble proteins cannot perform useful functions. In a Ca free system, the solubility of individual caseins is pH and temperature dependent (Bingham, 1971). The caseins are insoluble at their isoelectric point (pH 4.6) and the insolubility range becomes wider with increasing temperature (Fox & McSweeney, 1998). Due to the open structure and relatively high water binding capacity of caseins, they are able to form high viscous solutions; however, not more than 20 % casein protein can be dissolved even at elevated temperatures (Fox& McSweeney, 1998).

2.2.3.2 Heat stability

Caseins have remarkable heat stability because of the structure and contribution of κ− casein. Firstly, being amorphous unstructured proteins, the caseins do not denature and aggregate on heating (Fox & McSweeney, 1998). Secondly, the structure of casein micelles is such that the Ca sensitive caseins are located inside the micelles and are protected from further aggregation at elevated temperature by the outer layer of κ−casein (Griffin, et al., 1986).

2.2.3.3 Surface activity (or emulsifying capacity)

The surface activity of caseins is also an important property that makes them good foaming agents and emulsifiers (Fox & McSweeney, 1998). To be an effective emulsifying agent, a molecule should be relatively small and capable of adsorbing into an oil−water or air−water interface. In the milk, α_{s1}− and β−casein have relatively high surface hydrophobicity and meet this requirement very well (Dickinson, 1989). The more detailed information on the science of these properties will be discussed in the MPC functionality.

2.2.3.4 Co-precipitation with whey protein

Casein and whey protein co−precipitation can be achieved by acidification of denatured whey proteins to pH 4.6 or by addition of 0.2 M of $CaCl_2$ at 90 °C (Fox adsorbing 1998). The functionality of the caseins is not adversely affected when adjusting the milk to an alkaline pH before denaturing the whey proteins and then co−precipitating them with casein acidification at pH 4.6. This might be because the denatured whey proteins do not form complex with the casein micelles at elevated pH (Mulvihill, 1992).

2.2.3.5 Gelation

In milk, caseins undergo gelation when the environment is changed in one of several ways. The most important of these are rennet induced coagulation for cheese or rennet casein manufacture and acidification to the isoelectric point. In addition, caseins may also be gelled or coagulated by organic solvents or extremely severe thermal conditions (Fox adsorbing 1998). Studying the gelation process can provide crucial information on the casein micelle structure (Horne, 2002).

2.3 Milk Salts

Milk contains inorganic and organic salts. The concept of ‘salts’ thus is not equivalent to ‘mineral substances.’ Salts are by no means equivalent to ‘ash’ because ashing of milk causes loss of organic acids including citrate and acetate and because organic phosphorus and sulphur are transferred to

inorganic salts during ashing. The salts are only partly ionized. The organic acids occur largely as ions or as salts, citrate is the principle one. The mineral substances−primarily K, Na, Ca, Mg, Cl, and phosphate are not equivalent to the salts (Walstra, et al., 2006).

The salt composition varies, but the various components do not vary independently of each other. The dissolved salts affect various milk properties including protein stability. These salts are only present in the serum. Not all of the salts are dissolved, and not all of the dissolved salts are ionized. The casein micelles contain the undissolved salts. In addition to counterions of the negatively charged casein (mainly Ca, Mg, K, and Na), the micelles contain the CCP, which also contains some citrate. The colloidal phosphate is amorphous, can vary in composition, and may have ion exchange properties (Walstra, et al., 2006).

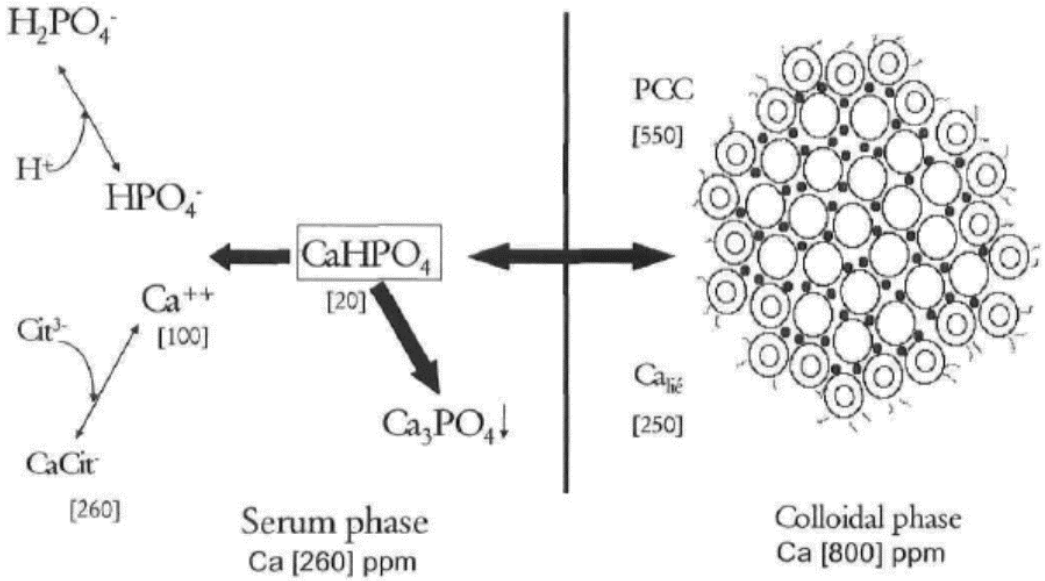


Figure 2.2. Salts equilibrium in milk (Britten and Pouliot, 2002).

Both the main cations of Ca, Mg, P and Na ions, and the dominant anions of phosphate, citrate and chloride partitioned between the colloidal and aqueous phase of milk, play an important role in the structure and stability of casein micelles in the milk (Holt, 1997; Walstra & Jenness, 1984). In normal milk, the distribution of Ca between aqueous and colloidal phase is balanced, and it

stabilises native micelle structure (Fox & McSweeney, 1998; Gaucheron, 2005). There is equilibrium between two phases (Figure 2.2). One is a dynamic and very rapidly attained equilibrium between the ion pairs and the free ions in the aqueous phase, and other is a dynamic but slowly attained equilibrium between salts in the aqueous phase and colloidal phase. The equilibrium can be broken or shifted by any physicochemical changes in the milk (Walstra & Jenness, 1984).

The minerals, in particular the Ca, play an important role in the integrity of the casein micelles (Holt, 1986). The role of Ca in chemical reactions, molecular arrangement with phosphorus and the effect on heat coagulation of proteins were well studied by White and Davies (1958a; 1958b) and Holt (1992). The formation of CCP, as an effective way of "burying" a considerable amount of Ca and phosphate within casein micelles, therefore largely determines the physicochemical properties of casein micelles (Lucey, 2003). About 70 % of total Ca and 30 % of total phosphate as principle colloidal salt are in the colloidal phase, which is naturally combined with four major caseins (α_{s1}-, α_{s2}-, β- and k-casein) in the form of aggregates in casein micelles which has been extensively reviewed by Fox & McSweeney (1998).

The chemical composition of CCP was first suggested by Pyne (1960) that CCP has an appetite structure with the formula: $3Ca_3(PO_4)_2 \cdot CaHCitr^-$ or $2.5Ca_3(PO_4)_2 \cdot CaHPO_4 \cdot 0.5Ca_3Citr^{2-}$. The hypothesis from Schmidt (1982) suggested that CCP is most likely to be amorphous Ca phosphate [$Ca_9(PO_4)_6$] ion clusters linked through Ca bridges to the phosphoryl residues of caseins. Based on this information, a more detailed physical study on the structure of CCP was undertaken. It was concluded that the CCP does not precipitate out of milk because of physical protection and chemical association with caseins inside the micelles and that it plays an important role in the structure and function of casein micelles (Fox & Hoynes, 1975; Rose, 1961; Singh & Fox, 1985; Singh & Fox, 1987). It was demonstrated that Ca either as free ions (Ca^{2+}) or ion complexes or in the colloidal form played an important role in the integrity of micelle and as bridge in between the soluble phase and colloidal phase (Holt, et al., 1986).

On average, cow's milk contains approximately 30 mM of total Ca, about 20

mM is associated with the casein micelle and 10 mM is soluble Ca, mostly as un−dissociated Ca phosphate (about 0.7 mM) or citrate (0.2 mM) as shown in Table 2.6. A small amount of it is present in ionised form and termed ionic Ca (Ca^{2+}). At the normal pH of milk, less than 10 % of total Ca is presented as ionic Ca (Holt, et al., 1981).

Table 2.6. Calcium partition in cow milk by Holt and Jenness (1984).

Component	Mean[1] value (mM)
Total Ca	29.4
Ionic Ca	2.0
Soluble Ca	9.2
% Soluble Ca	31 %
Micellar Ca	20.2
Micellar Ca / g of casein	0.77

[1] Mean values taking into account several breeds of cows.

Ionic equilibrium is known to play an important role in the structure and stability of casein micelles (Horne, 1998; Walstra, 1990). Alterations in the ionic equilibria induce significant changes to the ionic composition between the colloidal phase and aqueous phase. This will affect the physicochemical properties of casein micelles and the stability of products during processing and storage (De La Fuente, 1998; Fox & McSweeney, 1998; Huppertz & Fox, 2006).

The equilibrium between the soluble and colloidal salt of milk is influenced by many factors including changes in temperature, or concentration, and addition of salts. The details of these relationships and other important effects relating to mineral balance on the property and structure of casein micelles are now discussed below, in the context of alterations to casein micelles in response to environmental factors.

2.4 The Factors Influencing the Milk Salt and Casein Micelles Equilibrium

When milk is exposed to different environmental conditions such as temperature, pH and Ca chelating agents, the equilibrium between milk salt and micelles will change, the physicochemical properties of casein micelles will be altered and this will affect the properties of the final product.

2.4.1 THE EFFECT OF TEMPERATURE

Temperature has a large influence on the state of the milk system. As mentioned above, the caseins have marked heat stability and there are two main reasons for this. Firstly, being amorphous unstructured proteins the caseins do not denature and aggregate on heating (Fox & McSweeney, 1998) and secondly, the structure of casein micelles is such that the Ca sensitive caseins are located inside the micelles and are protected from further aggregation at elevated temperature by the outer layer of κ−casein (Griffin, et al., 1986). The high heat stability of caseins allows heat sterilized dairy products to be produced without major changes in physical properties. The heat stability of un−concentrated milk is almost always sufficient to withstand the normal heat treatment processes. However, the heat stability of milk decreases sharply on concentration (Fox & Brodkorb, 2008) and is usually inadequate to withstand in−container or UHT processing unless certain adjustments and / or treatment are made (Fox & Morrissey, 1977; Horne & Muir, 1990). Soluble casein is known to shift from serum into the micelles when increasing the temperature (Creamer, et al., 1977; Rose, 1968), which consequently leads to a compact internal micelle structure and decrease of the apparent voluminosity of casein micelles (Nöbel, et al., 2012). The effects of heat treatment on the partitioning of caseins between serum and micelle phase are reversible on cooling (Creamer, et al., 1977; Downey & Murphy, 1970; Pouliot, et al., 1989). The β−casein is able to shift from large micelles to serum (Creamer, et al., 1977) and κ−casein is solubilised from small micelles (Ono, et al., 1990) at low temperature. Loss of these temperature sensitive caseins from micelles leads to additional releases of caseins to serum (Rose, 1968). At high temperature,

interactions between denatured whey proteins and caseins can occur (Donato, et al., 2007; Jovanovic, et al., 2007; Oldfield, et al., 2000), due to the formation of inter−molecular disulfide bonds (Jang & Swaisgood, 1990; Lowe, et al., 2004). During heating, whey proteins denature, exposing free thiol groups that then form disulfide bonds with κ−casein on the surface of the casein micelles. This can result in a significant size increase as well as surface modification of the casein micelles (Anema & Li, 2003a; Park, et al., 1996; Singh, et al., 1997). The β−Lg is essential in this whey protein−casein reaction (Considine, et al., 2007; Corredig & Dalgleish, 1999; Elfagm & Wheelock, 1978). It forms aggregates with α−La in a fixed ratio before interacting with caseins (Guyomarc'h, et al., 2003). The addition of β−Lg reduces the level of soluble α− and β−casein at temperatures above 60 °C (Anema, et al., 2000), presumably due to the formation of whey protein - casein complex.

The exchange of minerals between the serum and colloidal phases is also temperature dependent and soluble Ca concentration is known to decrease at high temperature (Rose & Tessier, 1959) due to decrease of solubility (Morr, 1967b). Cooling on the other hand, increases the concentrations of soluble Ca and phosphate at the expense of CCP (Fox & McSweeney, 1998; Pouliot, et al., 1989). The mineral composition of milk is also important to the zeta potential of casein micelles, which decreases as a function of temperature (Darling & Dickson, 1979 a, 1979b).

2.4.2 THE EFFECT OF pH

Casein micelles formed at low pH have more compact structure due to low charge on the proteins (Liu & Guo, 2008). The composition and surface hydrophobicity of micelles can be changed by lowering the pH (Jean, et al., 2006; Law, 1996). A progressive solubilisation of CCP and other colloidal salts from the micelles takes place at low pH regardless of the overall ionic strength (Le Graët & Gaucheron, 1999). Most of the remaining Ca ions are solubilised when pH reaches 4.6, with the disappearance of the CCP nano−clusters confirmed by small angle X−ray scattering (Marchin, et al., 2007).

Casein micelles have a sufficiently open structure to allow movement of casein proteins and the exchange of mineral ions (Udabage, et al., 2003). The

voluminosity of the core of the casein micelles is dependent on the hydration and relative partitioning of exchangeable minerals and casein between the micelles and serum. The overall (hydrodynamic) voluminosity of the casein micelles also depends on the thickness of the κ−casein hairy layer, which increase with the addition of NaCl (Kapsimalis & Zall, 1981). Adding Ca has more complicated effects. It increases the Ca activity (McGookin & Augustin, 1991) which decreases the soluble casein concentration (Famelart, et al., 1999; Sood, et al., 1979). It also reduces the negative charge of caseins (Anema & Klostermeyer, 1997; Anema, et al., 2005) and weakens the κ−casein hairy layer (Müller-Buschbaum, et al., 2007) thereby modifying the hydrophobic interactions and electrostatic interactions between casein micelles (Van Hooydonk, et al., 1986).

2.4.3 THE EFFECT OF Ca CHELATORS

Calcium chelators are organic compounds that bind to Ca ions forming a complex. The CCP can also be removed from casein micelles by adding Ca chelators, such as Ethylenediaminetetraacetic acid (EDTA), citrate or oxalate (Horne, 2002), which might combine with the Ca and release of soluble caseins (Griffin, et al., 1988; Lin, et al., 1972). Adding citrate reduces the concentration of CCP, and increases the soluble Ca, soluble phosphate and the pH (Fox & McSweeney, 1998).

It was reported that addition of Ca chelators will increase the repulsion between the negatively charged amino acids in the casein micelles, resulting in an increase in hydration and voluminosity of the micelles (Gaucheron, 2005; Walstra, 1979), decrease in turbidity of milk (Walstra, 1990; Panouille, et al., 2004), and increase in viscosity through interactions with the casein proteins when Ca chelators are added (Augustin & Clarke, 1990; Singh, et al., 1995; Holt, 1992). The micelles eventually dissociate into small clusters and dispersed proteins at higher chelator levels.

The extent to which chelators affect the micellar structure depends on their Ca−binding capacity (De Kort, et al., 2009) and their interaction with the Ca ions and amino acids in the casein micelle (Mizuno & Lucey, 2007) and the amount or concentration of chelators (Keowmaneechai & McClements, 2002). Besides

the generic property of Ca−binding, Ca chelators may generate specific effects, such as increased heat stability or retard age gelation in dairy products (Leviton & Pallansch, 1962; Augustin & Clarke, 1990; Singh, et al., 1995). Panouillé, et al. (2005) and Pitkowski, et al. (2008) have shown that intact and dissociated casein micelles can be present simultaneously in the milk after addition of chelators. Kaliappan and Lucey (2011) reported that when phosphates and citrates were added to milk system the heat stability was improved. Sodium salts of phosphoric or citrates were also added to increase the heat stability of concentrated or sterilized milk by Sweetsur and Muir (1980) and to decrease the susceptibility of ultra−high temperature processed milk products to age−induced gelation (Walstra, 1999). Ca chelating salts (CCS) were also used in the manufacture of processed cheese (Zehren & Nusbaum, 1992; Caric & Kalab, 1993).

2.4.4 CASEIN MICELLE ALTERATIONS DURING UF

UF is an important processing step in the manufacture of cheese and dairy ingredients and in milk standardization (Gésan−Guiziou, 2013). During UF of skim milk, water, soluble minerals and lactose pass though the membrane into permeate while proteins are concentrated in the retentate (Mistry & Maubois, 2004).

The casein micelles have been reported to be responsible for concentration polarization and gel layer that form on membranes during UF of skim milk, directly influencing flux and processing efficiency (Bouzid, et al., 2008; Rabiller−Baudry, et al., 2005). High zeta potential of the casein micelles will lead to high processing flux at neutral pH (Bouzid, et al., 2008; Rabiller−Baudry, et al., 2005). In the meantime, casein micelles are dynamic entities that can themselves be affected by UF processing. A fundamental understanding of casein micelle behaviour during UF, could therefore lead to improvements in processing efficiency (David, et al., 2008; Rabiller−Baudry, et al., 2005), the use of UF retentates for standardizing cheese milk, and the quality of products such as MPC powders (Martin, et al., 2010). Despite this, to date only a limited number of studies have been devoted to understanding the effect of UF on the composition, size or structure of casein micelles (Singh, 2007).

In one analysis using transmission electron microscopy (TEM) images, a decreased average casein micelle size was observed in UF retentates at volume concentration factors (VCF) of 3 and 5 compared with the un−concentrated skim milk (Srilaorkul, et al., 1991). The size change was attributed to compositional changes in the retentate; however the composition of the micelles was not directly investigated. In another study employing TEM, the size of casein micelles was observed to remain relatively unchanged during UF but become progressively swollen on subsequent diafiltration (McKenna, 2000). In a more recent study, different methods were employed to compare casein micelles in fresh skim milk to those in commercial UF retentates and reconstituted MPC powders. The average size of casein micelles was found to be similar in all these samples as measured by dynamic light scattering (Martin, et al., 2010). Rabiller−Baudry, et al. (2005) and Karlsson, et al. (2007) have investigated the effect of pH and mineral content on the physical properties of skim milk during membrane filtration and found that the casein micelle voluminosity appeared to increase in the presence of added NaCl and decrease when the pH was decreased from 6.5 to 5.5. In studies on the functional properties of UF treated milk, alterations to the bulk mineral composition caused by UF have been shown to affect the renneting process (Ferrer, et al., 2011; Martin, et al., 2010; Sandra & Corredig, 2013). While these studies showed that UF processing can affect the properties of casein micelles, alterations to the composition of the casein micelles were not investigated. In addition, as discussed above, the temperature effects on casein micelles are very important. This information will lead to a better understanding of UF processing of skim milk and the properties of the resulting retentates. Moreover, during UF processing, milk can be highly concentrated and the casein micelles still behave like a dispersion of hard spheres (Bouchoux, et al., 2009; Mezzenga, et al., 2005; Sandra, et al., 2011). However, further concentration (casein concentrations up to 500 g / L, which is well above the casein concentration within a micelle; 230 g / L taking a voluminosity of 4.4 mL / g) leads to expulsion of the internal water and micelle fusion (Bouchoux, et al., 2009).

2.5 Milk Protein Concentrate (MPC)

There are many different grades and types of protein−enriched products, e.g., caseins and caseinates, whey protein concentrates (WPCs), whey protein isolates (WPIs), and MPCs, the specific protein blends designed for particular applications. In this study, the MPC is the focus and therefore the review is limited to MPC. As per the FDA, MPC should contain all of the proteins and in the same ratios that are naturally found in milk (AOAC 1998; Garde, 2008). Thus MPCs are complete milk proteins, which contain both caseins and whey proteins, produced by drying the concentrates. The MPC is available in protein concentrations ranging from 40 to 89 % as shown in Table 2.7. It can be seen that as the protein content of MPCs increases, the lactose levels decreases.

Table 2.7. The composition of some MPC (Garde, 2008).

Constituents	Skim milk powder	MPC 42	MPC 56	MPC 70	MPC 80
Moisture	3.2	3.5	3.8	4.2	3.9
Fat	0.8	1.0	1.2	1.4	1.8
Protein	36	42	56	70	82.8
Lactose	52	46	31	16.2	4.1
Ash	8	7.5	8	8.2	7.4

Note: Values written as in per cent

MPC is significantly different from WPC which is concentrated from cheese whey. Since cow's milk contains approximately 80 – 82 % casein and only 18 − 20 % whey proteins, it is easy to understand that MPC contains only a small amount of whey protein. Whilst WPC on the other hand, contains whey protein as sole protein, there is no casein present in WPC. MPC forms a milk white suspension when dispersed in water. WPC (and WPI) form somewhat clear, brownish tinted dispersions in water. Aqueous dispersions of MPC have a bland or creamy flavour. Aqueous dispersions of WPC tend to have a slightly astringent flavour due to the high levels of sodium, potassium, and chlorine (Pellegrino, et al., 2013). Therefore MPC and WPC or WPI are significantly different in all respects−nutritionally, compositionally, and functionally as well as the production process. MPC powders can be used to replace whey proteins in

nutritional applications without any issue of functionality and organoleptic (Pellegrino, et al., 2013).

2.5.1 MPC MANUFACTURING

The process used in dairy industry for producing MPC powder is shown in Figure 2.3. The MPC powder is produced by further processes of evaporation and drying of the retentate obtained after UF / DF.

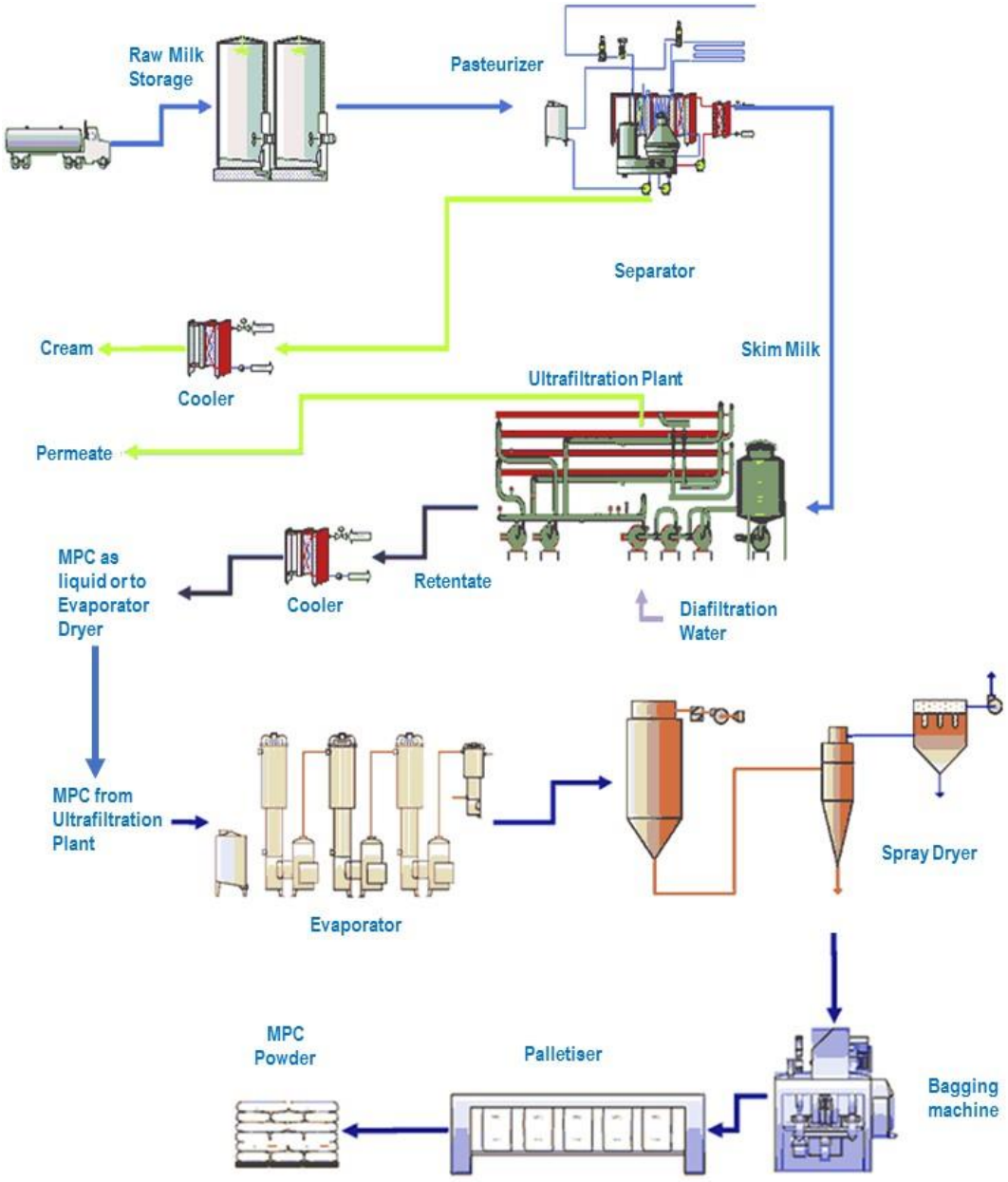


Figure 2.3. MPC and MPC powder processing diagram (GEA Filtration, GEAF Process Engineering Inc., Hudson, WI; USA).

2.5.2 MEMBRANE TECHNOLOGIES FOR MPC MANUFACTURING

In milk, there are two clear groups of compounds based on size. Minerals and lactose are small in size while proteins, including casein and whey proteins, are much larger. It is this large difference between the sizes of the two groups of components that allows milk to be separated efficiently using membranes.

Membrane filtration was developed in the 1970s by the pioneering work of Professor Maubois and colleagues in France (Maubois & Mocquot, 1975; Maubois, 1991), which is a sieving process that separates milk components according to their size and determines the composition of the MPC, while evaporation and drying are used to remove only water from the concentrates produced. The relationship between sizes of milk components and pore size of filtration membranes is illustrated in Figure 2.4.

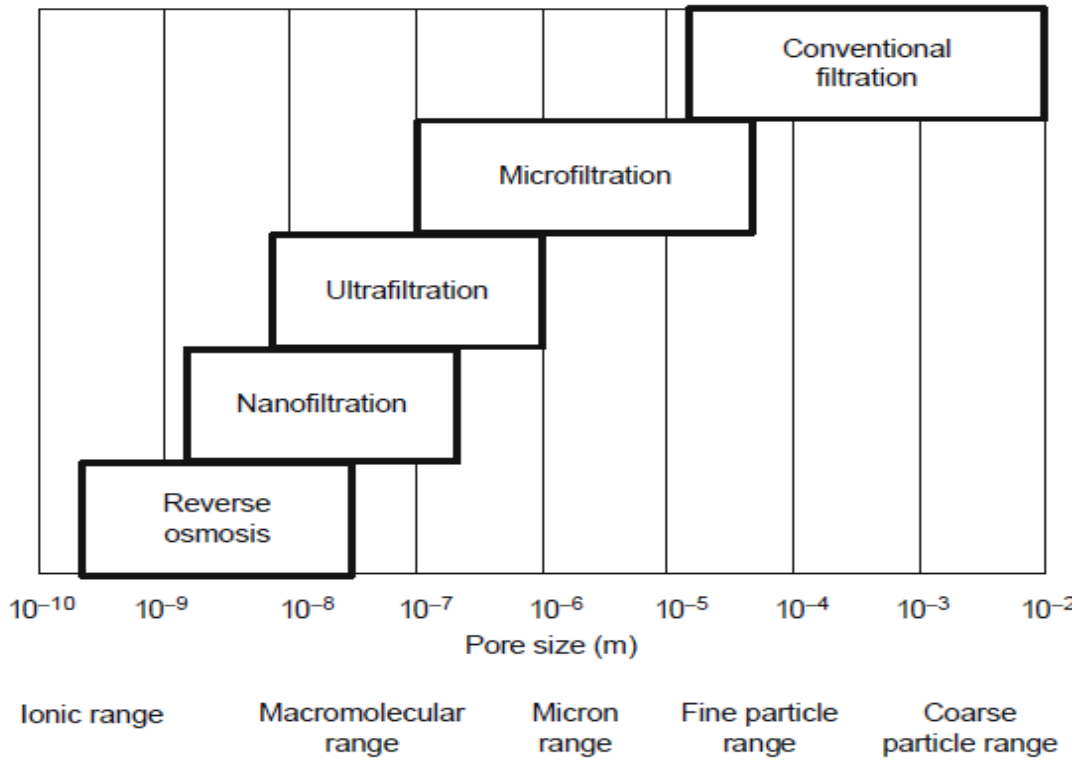


Figure 2.4. Relative milk components sizes in comparison with membrane pore size ranges (adapted from Dairy Processing Handbook, 2003)

Microfiltration (MF) is primarily used for removing bacteria from skim milk, whey and brine, although sometimes it is also used for whey defatting.

Ultrafiltration (UF) is typically used for concentration and standardization of milk proteins which is intended for cheese, yoghurt and other products. It is also used for clarification of fruit and berry juices (Dairy Processing Handbook, 2003).

Nanofiltration (NF) is used for desalination of whey, UF permeates or retentate by partial removal of monovalent ions like sodium and chlorine.

Reverse Osmosis (RO) is used for dehydration of whey, UF permeates and retentates by removal of water.

This review will primarily focus on UF.

During UF, milk passes across a membrane that resembles a piece of thin plastic. Some of the lactose, minerals and water will cross through the membrane and become the permeate stream (Figure 2.5).

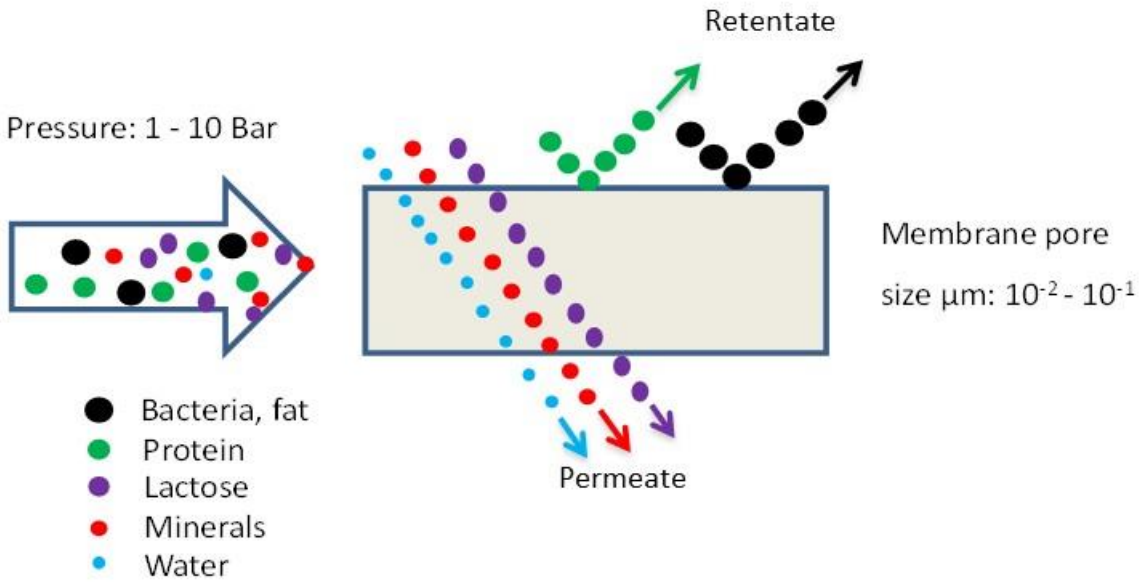


Figure 2.5. The principles of UF membrane filtration (adapted from Dairy Processing Handbook, 2003)

The permeate thus obtained is an aqueous solution of lactose, minerals, non-protein nitrogen, vitamins and other low molecular weight compounds. Casein and whey proteins, because of their large size, will not be able to pass through the membrane. The proteins along with the lactose and minerals that did not go into the permeate stream will become the retentate stream. The concentration of protein in the retentate stream (with normal range of 42 − 80 % dry base) will

increase as more lactose and minerals are removed in the permeate stream (Rattray & Jelen, 1996).

A diafiltration (DF) sometime called “washing step” is required after UF to get protein concentration greater than 65 % in the final dried product. DF involves adding water to the retentate as it is being ultrafiltered to reduce product viscosity and further remove lactose and minerals. The DF can be conducted by either one of the two modes: discontinuous or continuous DF (Walstra, et al., 2006).

The degree to which the retained species has been purified is normally presented in terms of the volume concentration ratio (VCR) (Walstra, et al., 2006):

$$VCR = \frac{Initial\ volume\ (V_0)}{Retentate\ volume\ (V_R)}$$

VCR is also referred to as “Volume Concentration Factor” (VCF).

Following UF or UF / DF, the retentate is evaporated to increase the total solids in the processing stream which improves dryer performance. The common drying method used for drying of retentate is spray drying for industry or freeze drying for small lab scale.

Falling film tubular evaporators are typically used in the spray drying for the concentrates because milk proteins are sensitive to damage by heating. Dryer designs such as tall form spray bed and stage dryers are considered appropriate for drying MPC. These types of dryers are designed to minimize the temperature exposure of the MPC during drying so that important functional properties such as solubility are retained (Jimenez−Flores & Kosikowski, 1986).

Freeze drying is based on the fundamental principle of sublimation, the shift from a solid directly into a gas. Just like evaporation, sublimation occurs when a molecule gains enough energy to break free from the molecules around it. Water will sublime from a solid (ice) to a gas (vapour) when the molecules have enough energy to break free but the conditions aren't right for a liquid to form. In

this project freeze drying was used for producing MPC powder.

The main advantage of freeze−drying is that once the water is removed from foods, they become very light. This makes for easier portability of large amounts of food and cheaper transportation of the food. Additionally, freeze−dried foods tend to retain most of their nutritional quality, taste, shape and size. Freeze dried MPC powder do not require refrigeration, and can last for months or years. Freeze−dried foods can also be rehydrated very quickly, unlike other types of dehydrated foods (Holdsworth, 1971).

2.5.3 THE ADVANTAGE OF MEMBRANE FILTRATION

Introduction of membrane technology into dairy processing witnessed a phenomenon of mutual benefit for membrane technology as well as for dairy industry. The marked improvement in the nutritive quality and sensory attributes of the existing dairy products with higher yields in addition to development of several innovative dairy products became possible by the application of membrane technology. The significant advantages of membrane separation processes over competing approaches of concentration or separations are becoming increasingly important in the food and biotechnology industries, and include:

- No phase or state change of the solvent is required — more cost−effective.
- Operation is usually at relatively low temperatures — therefore, they are suitable for processing thermolabile materials, reducing changes in flavour or other quality characteristics, and minimizing heat denaturation of enzymes.
- Good levels of separation can be achieved without the need for complicated heat transfer or heat-generating equipment.
- Separations cover a wide spectrum of sizes, ranging over several orders of magnitude, from the smallest ions to particles such as fat globules or bacterial cells.

2.5.4 THE MAJOR PROBLEMS OF MEMBRANE TECHNOLOGY

A limitation of membrane processes is that they cannot concentrate solutes to dryness. The degree of concentration is limited by the extreme osmotic pressures, high viscosities or low mass-transfer rates generated at the increased solute concentrations (Cheryan, 1998).

The major problem of membrane filtration is the membrane fouling presented as permeate flux declining with the progress of concentration. Membrane fouling can occur due to blockage of membrane pores, adsorption of particles on the pores, deposition of protein and minerals, cake formation and depth fouling (James, et al., 2003; Popovic, et al., 2010; Anand, et al., 2012), and bacterial biofilms (Kumar & Anand, 1998; Tang, et al., 2009). The fouling caused by the deposition and accumulation of particles, bacteria and sediments of feed component present in milk on the membrane surface and /or within the pores of the membrane, causes an irreversible flux decline during processing, leading to remarkable loss in the efficiency of membranes. The fouling of membrane remains the major concern in dairy industry (Gesan, et al., 1995; Makerdij, et al., 1999). Membrane fouling is influenced by casein micelle size, membrane surface chemistry and ionic strength, temperature, pH, feed flow and viscosity of feed stream and the problem can be addressed by various techniques such as back pulsing, cross flushing and backwashing, (Huimin, et al., 2001).

Fouling during UF manifests itself as a decrease in flux over time due to higher thermal resistance and temperature polarization, and reduced area and increased tortuosity for mass transfer. It is a result of specific interactions between the membrane and various solutes in the feed−stream, and between the absorbed solutes and other solutes in the solution as well as compaction of the deposited layer upon interaction between components in that layer (Bottino, et al., 2000; Tolkach & Kulozik, 2006). Each component of a feed−stream will interact differently with the membrane. Protein conformation, zeta potential, and other factors will influence these membranes−solute interactions, and it is expected that the chemistry of the dairy components will dictate the ultimate fouling mechanisms in membrane filtration process of dairy applications (Hausmann, et al., 2013).

Dairy fouling studies to date have primarily focused on the more common pressure driven skim milk filtration, however detailed information on membrane performance (e.g. permeate flux decline, surface fouling and internal structure deformation) during skim milk filtration with change of milk environment (temperature, pH and with addition of Ca chelators) is limited and need be further studied.

2.5.5 MEMBRANE DESIGN

Membrane filtration can be designed to be (A) cross flow filtration or (B) dead−end filtration as shown in Figure 2.6. Cross−flow filtration gets its name because the majority of the feed flow travels tangentially across the surface of the filter (Figure 2.6A), rather than into the filter. For the dead−end filtration, the feed is passed through a membrane or bed (Figure 2.6B), and the solids are trapped in the filter and the filtrate is released at the other end.

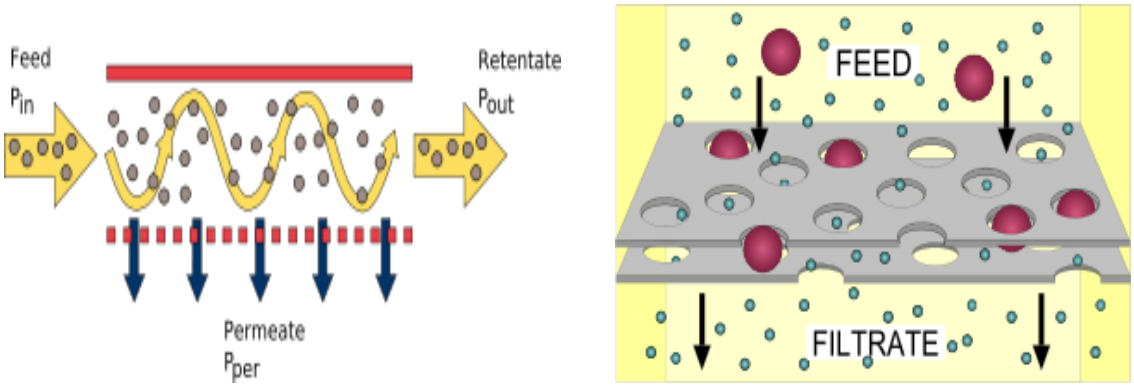


Figure 2.6. Membrane filtration configurations: (A) cross flow (https://commons.wikimedia.org/wiki/File:Cross−flow.jpg) and (B) dead end filtration (https://en.wikipedia.org/wiki/Filtration).

Membrane materials vary from Polyethersulfone, ceramics, and metal, etc. The polyethersulfone (PES) is a heat−resistant, transparent, amber, non−crystalline engineering plastic having the molecular structure comprises of phenylene rings connected by alternate sulfonyl (SO_2) functional groups and ether (–O−)

linkages (Figure 2.7). This sulfonyl groups confer rigidity (with a high glass transition temperature) and, together with the ring structures, chemical resistance and relative hydrophobicity. The ether linkages make the polymer less hydrophobic and more flexible, hence more amenable to processing.

PES is a tough and rigid resin similar to conventional engineering plastics, such as polycarbonate, at room temperature. The greatest characteristic of PES is that it has by far greater heat resistance property than conventional engineering plastics. Specifically, PES remains in satisfactory condition in long−term continuous use without causing any dimensional change or physical deterioration at temperatures as high as 200 °C. PES membranes are hydrophilic filters constructed from pure polyethersulfone and designed to remove particulates during general filtration. Their low protein and drug binding characteristics also make PES membrane ideally suited for use in life science applications (Tamime, 2009).

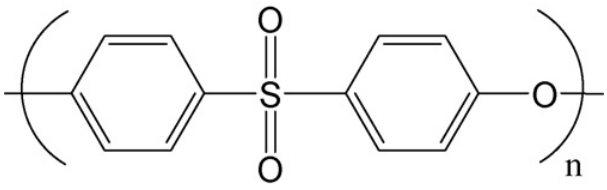


Figure 2.7. Molecular structure of polyethersulfone

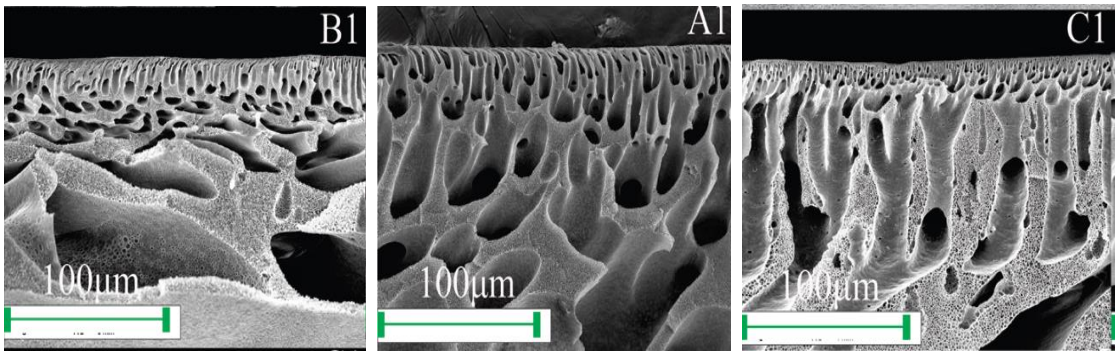


Figure 2.8. SEM images of membrane cross sections. A1, B1, and C1 represent PES molecular weights of 120, 65, and 42 kDa, respectively (Zhou, et al., 2010).

Figure 2.8 shows the SEM images of the PES membrane cross sections (Zhou, et al., 2010), which display the expected typical asymmetric structure with a thin dense skin layer and a thick porous sublayer consisting of two types of macrovoids, fingerlike and irregular and pore walls with dense spongelike structure. For a given MWCO (molecular weight cut off) of PES membrane, with increasing PES concentration, the fingerlike macrovoids to the skin layer become finer, longer, and more in number, while irregular macrovoids become less in size and the thicknesses of the walls between macrovoids increase.

2.6 The Factors Influencing MPC Production

There are many factors influencing the MPC production. Among them, the temperature, pH and addition of Ca chelating agents are the major ones, which were investigated in this study and reviewed here below.

2.6.1 TEMPERATURE

Temperature is an important parameter for skim milk concentration by UF process. UF at high temperatures above 70 °C (Lalande & Tissier, 1985) favours membrane filtration by lowering liquid viscosity (Pace, et al., 1976; ST−Gelais, et al., 1992) and increasing mass transport across the membrane (Marcelo & Rizvi, 2008). However, not only is product quality affected by the heat−induced reactions, but fouling of membrane by deposit formation on the membrane bed is governed by specific reactions of milk components, reducing the membrane performance. A high temperature could accelerate membrane fouling caused by protein denaturation leading to protein interaction and gelling on membrane surfaces (Gautam, 1994).

The effect of heat on protein denaturation and aggregation has been studied extensively and has been well reviewed (Considine, et al., 2007; Raikos, 2010). However, the investigation of MPC properties as affected by casein micelle size and heat mainly focused on using reconstituted MPC powders (Anema & Li, 2003a, b). The change of the casein micelles size induced by different heating conditions was reported by using centrifuged skim milk (Ono, et al., 1999). The

production of MPC at 10 and 50 °C has been reported by Havea (2006) and Syrios, et al. (2011) but the effect on the functional properties of MPC has not been studied. Consequently, comprehensive information on the effect of temperature of UF processing on the size of casein micelles and their functional properties is scarce.

2.6.2 pH

Change in pH has been found to influence the amount of minerals in the final retentate during UF of whey and buttermilk (Hiddink, et al., 1978). The shift in the casein−mineral equilibrium led to an increase / decrease in concentration of free Ca ions, dissolution of CCP from the micelle, release of specific casein from the micelle (Holt, 1992; Gaucheron, 2005; Panouille, et al., 2004; Griffin, et al., 1988) and alteration of casein micelle size.

There was no universal agreement on the effect of UF on casein micelle size during manufacturing of MPC powders. The size of casein micelles was reported to be unchanged (Karlsson, et al., 2007), increased (Singh, 2007; Srilaorkul, et al., 1991) or decreased (Erdem & Yuksel, 2006) as a result of UF. Martin, et al. (2010) concluded that there was no major change in the micelle size during UF / diafiltration or evaporation. Recently a report provided by Gregory, et al. (2010) suggested that there is no evidence on alteration to the size of micelles occurring during the UF / DF using standard industrial membrane UF system for raw skim milk, but changes are likely when related to changes in pH, Ca and phosphate equilibrium and milk concentration. However, the information of acidified skim milk after UF process and their related functionality is also limited. Therefore an investigation on the effect of UF process and change of pH on the casein micelles of MPC is necessary.

2.6.3 ADDITION OF CHELATORS

Calcium chelators such as citrate, phosphate, hexametaphosphate (HMP), or ethylenediaminetetraacetic acid (EDTA) are commonly used in dairy industry to improve the heat stability or to retard age gelation in dairy products (Augustin & Clarke, 1990; Harwalkar, 1982; Holt, 1985; Singh, et al., 1995). It is able to

induce various physical changes in the casein micelle by shifting the protein−mineral equilibrium, leading to a decrease in the concentration of free Ca ions, depletion of CCP from the micelles and release of specific caseins from the micelle (Holt, 1992; Gaucheron, 2005; Panouille, et al., 2004).

The effect of EDTA on the physical changes of the casein micelle in skim milk has been extensively studied (Lin, et al., 1972; Griffin, et al., 1988; Ward, et al., 1997; Udabage, et al., 2000). EDTA was known to be effective for the dissociation of casein micelles (Lin, et al., 1972). Information is also available on the effects of phosphates and citrate on the voluminosity of the casein micelle and turbidity of the milk (Leviton & Pallansch, 1962; Vujicic, et al., 1968; Mizuno & Lucey, 2005). The effect of citrate on physical changes of milk has been studied in skim milk systems, with low concentration factors (protein ~6.5 %, w/v), and relatively low chelator levels mainly focused on the formation of milk gels (Mizuno & Lucey, 2005) or on age gelation after addition of chelators (Kocak & Zadow, 1985).

Udabage, et al. (2000; 2001) investigated the effect of EDTA (5, 10 or 20 mM) or citrate (5,or 10 mM)at pH 6.65 on casein micelles, and found that the addition of citrate or EDTA, which removed more than 33 % of the original CCP with accompanying release of 20 % casein from the micelle, completely inhibited gelation. However, the solubility and other functionality regarding the change on the casein micelles size induced by shifts of protein−mineral equilibrium have not been reported. The information on whether further shifts induced by Ca^{2+} partial permeation / removal before or during UF processing is scarce. The different protein concentrations that affect functionality, and the comparison between EDTA and citrate acid are scarce.

2.6.4 OTHER FACTORS

Other factors that influence the MPC production include the addition of NaCl. Addition of NaCl to milk leads to a slight decrease in pH and increase in Ca^{2+} concentrations in the diffusible phase. These changes would correspond to exchanges of divalent cations or protons which were attached directly to phosphoryl residues of casein molecules by Na^{+} (Le Graët & Brulé, 1993; Le Ray, et al., 1998; Gaucheron, et al., 1996). The addition of divalent cations

(especially Ca) also induces important change in the salt distribution between aqueous and micellar phases. These changes depend on the type of cation (Ca, magnesium, iron, zinc, copper), the concentration at which they are added and the eventual modification and regulation of pH consequent to cation addition (Philippe, et al., 2005). It has been reported (Ichilczyk−Leone, et al., 1981) that in cooled skim milk one part of micellar Ca phosphate is transferred to the diffusible fraction within 24 h with increase of about 10 % of Ca and inorganic phosphate in the diffusible fraction. These changes are reversible and the previous partition may be re−established on re-warming.

2.7 The Functional Properties of MPC

Developments over recent years have resulted in MPCs of different compositions being produced and incorporated into a wide range of products. Application and formulation dictate the type of MPC used. Typically, low−protein MPCs are used as ingredients in cheese applications, while high−protein MPCs are used in beverage and bar preparations. They are used as an ingredient in the kind of applications that rely on non-fat dry milk / skim milk powder but require a higher level of protein where casein and caseinate are used traditionally. For example, MPC powders are often used to standardise the protein content in normal milk, and are used in the recombined cheese industry (Anema, et al., 2006). MPCs are currently also used for manufacturing various products including cheese, ice cream, yoghurt / fermented dairy products and meal replacement beverages as well. Use of MPCs as an ingredient is growing at the expense of casein and caseinate, due to their improved flavour profile. Common applications include infant formulas, desserts, baked goods, toppings, low−fat spreads, dairy−based dry mixes, dairy−based beverages, sports and nutrition beverages / foods, geriatric nutritional products, weight loss beverages / foods and some cheese products.

To be useful as functional ingredients in food products, MPC should exhibit good functionalities such as high solubility, heat stability and emulsifying capacity.

2.7.1 SOLUBILITY

Among the functional properties, solubility (i.e., rehydration capacity) of MPC powder is of primary importance being a fundamental prerequisite for other functionalities such as emulsification, gelation, and foaming. Mulvihill and Fox (1989) defined protein solubility as “the amount of protein in a sample that goes into solution or into colloidal dispersion under specified conditions that is not regimented by low centrifugal forces.” MPCs are desired to exhibit a good solubility for 6 to 8 months when stored at ambient to cool temperatures (4 to 21 °C) and low humidity conditions. However, whether solubility of higher−protein MPCs decreases over time or not depends on the intermolecular electrostatic interactions, hydrophobic interactions, and molar mass (Damodaran & Paraf, 1997; Walstra, et al., 2006). MPC powders with 40 to 90 % (wt / wt) protein content can have poor solubility, which restricts their potential food applications (De Castro−Morel & Harper, 2002; Fang, et al., 2011). Therefore, improving the solubility of MPC powder is essential for enhancing its functionality and use.

Although the solubility of MPC is an important functional property, only a few studies have been found in the literature; which could be due to the fact that MPC is a relatively new dairy ingredient (Anema, et al., 2006). Some protein concentrates are either naturally insoluble or rendered insoluble by processing treatments. Other protein concentrates tend to be insoluble at acidic pH value near the isoelectric point (pI) (Zayas, 1997). The major factors that influence protein solubility are pH, temperature, ionic strength and protein concentration (Vojdani, 1996).

Many researchers have tried to increase protein solubility by different techniques. Zwijgers (1992) found that the solubility of MPC could be improved by raising the temperature of water (from 20 to 50 °C) for MPC reconstitution, resulting in an increase in water transfer towards the interior of the powder particles. Babella (1989) found that increasing the Ca to total mineral ratio decreased the solubility of MPC. This was confirmed by Ye (2011), who showed that a reduction in Ca content resulted in an improved functionality of the MPC. However, an increase in the K or Na to total mineral ratio did not change its solubility. This fact was challenged in a more recent study that found

that the solubility of MPCs increased with increasing Na or K content (Sikand, et al., 2011). The enhanced solubility of MPC 80 powder with addition of NaCl during diafiltration may result from the modified surface hydrophobicity, the reduced intermolecular disulfide bonds, and the associated decrease in mean casein micelle size as reported by Mao, et al. (2012). Recently, de Castro−Morel and Harper (2002) collected samples of 37 different MPC products from 10 different countries to examine their basic functionality. They found no correlation between protein content and solubility, viscosity or foaming, except for a small sub−group of the MPC powders with high protein levels (82 − 86 %) where a correlation between solubility and protein content, pH and viscosity was found. De Castro−Morel and Harper (2002) indicated that the variability in the functional properties of MPC, and in particular the variable solubility, could impact negatively on MPC achieving full market potential. Therefore an improvement in solubility makes MPCs more suitable for use in specific food and beverage applications such as meal replacement beverages and processed cheese. Certain mineral such as Ca, Mg and P are known to interfere with protein solubility and thus provides food manufacturers with techniques to control and customize the mineral content of their dairy powders for use in specific food and beverage applications (Zwijgers, 1992; Schuck, et al., 2002; Hussain, et al., 2011; Sikand, et al. 2012; Mao, et al., 2012).

It is widely believed that the processes involved in manufacture of MPC maintain the original state of casein micelles (Mulvihill & Ennis, 2003). The delicate ionic equilibrium between serum and micelles in milk (Holt, et al., 1981) is shifted during membrane filtration. This change may cause a lasting effect on the casein micelles, affecting their rehydration and the functional properties of the resulting concentrates. Other factors such as pH, temperature and protein concentration during MPC production also influence protein solubility (Vojdani, 1996).

2.7.2 HEAT STABILITY

The heat stability of milk powder has drawn much attention and has been extensively studied over the past 40 years (Fox & Morrissey, 1977; Fox, 1981; Singh, et al., 1995, 1997; Van Boekel, et al., 1989; Singh & Creamer, 1992;

O'Connell & Fox, 2003).

The heat stability of MPC powders have been the focus of a few studies (Anema, et al., 2006; Fang, et al., 2011; Havea, 2006). It was found that the formation of complexes between β−Lg and κ−casein, in the serum or colloidal phases, is associated with regions of minimum and maximum heat stability in milk, respectively (Oldfield, et al., 2000; Rose, 1961). The production of organic acids (mainly formic) through heat−induced degradation of lactose (Fox, 1981; O'Brien, 2009) and high Ca−ion activity (Philippe, et al., 2003; Sievanen, et al., 2008) has negative effects on heat stability of MPC. Reduced levels of phosphate cause a shift in regions of heat stability to more alkaline pH values (O'Connell & Fox, 2003; Rose, 1961). In addition, phosphates, as well as other Ca−binding salts, are sometimes added to milk to improve heat stability, due to their ability to increase buffering capacity and reduce Ca−ion activity (De Kort, et al., 2012).

More recently Crowley (2014) investigated the heat stability of MPC powders in range of 36.6 % (w/w; MPC 35) to 89.6 % (w/w; MPC 90) protein in dry matter at 140 °C in the pH range 6.3 − 7.3. It was found that at pH < 6.8, the heat stability of MPC suspensions decreased with increasing protein content of the MPC powders, due to high Ca−ion activity. At pH > 6.8, the influence of increased Ca−ion activity with increasing protein content of the MPC powders was countered partially by reduced heat−induced κ−casein dissociation. The heat stability of MPC 80 was restored by re−establishment of the serum composition of skim milk; fortification with lactose or urea only affected heat stability outside the pH region where rapid Ca−induced coagulation occurred.

In various applications, MPC powders are likely to be reconstituted and subjected to heat treatments such as ultra−high temperature processing or retort sterilisation; thus, it is important to determine the heat stability of reconstituted MPC powders to prevent process variability or loss in product quality due to heat−induced destabilisation. However, the heat stability of reconstituted MPC powders with pH adjusted and addition of Ca chelators has not yet been studied.

2.7.3 EMULSIFYING PROPERTIES

One of the primary functionality requirements of several food systems is the ability to form emulsions. An emulsion is defined as a dispersion or suspension of two immiscible liquids but the structure of most food emulsions is much more complex (Dickinson 2008). Emulsion can be produced as oil−in−water (o/w), droplets of oil are suspended in an aqueous continuous phase, like milk, and water−in−oil (w/o) emulsions which are typified by butter, margarines and fat based spreads in general (Dickinson, 1999; Dalgleish, 2006).

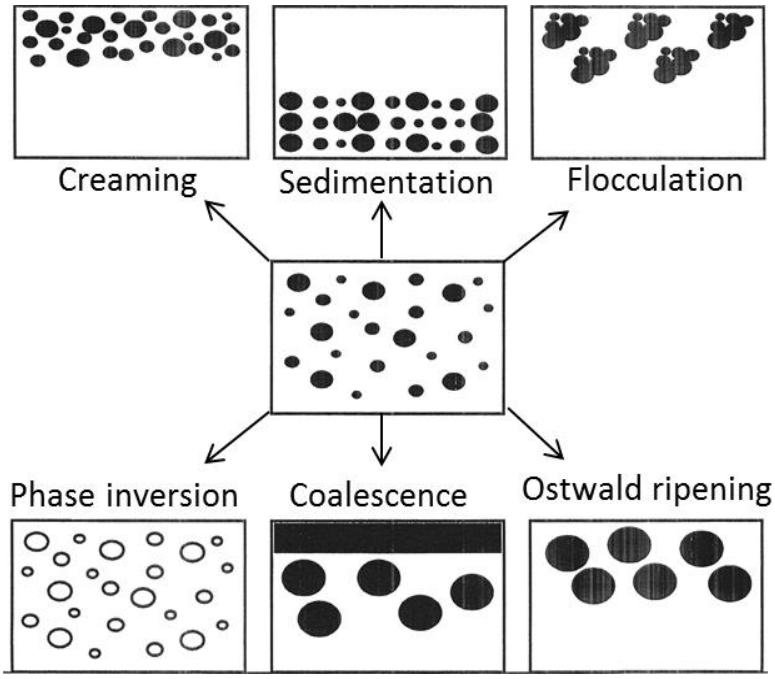
As food ingredients, MPC, in both soluble and dispersed form, have excellent emulsifying and emulsion stabilising characteristics: acting as an emulsifier during homogenisation, forming individual molecules or protein aggregates and being rapidly adsorbed at the surface of the newly formed oil droplets. These steric stabilising protein layers protect the fine droplets against immediate recoalescence and provide long−term physical stability to the emulsion during subsequent processing and storage (Dickinson, 1997).

Emulsifying properties of proteins are important parameters to determine their functional properties under different environmental conditions and the possible interaction with other macromolecules present in the media. Emulsion capacity is the parameter most commonly estimated in the various studies on oil−in−water emulsions (Hill, 1996). Emulsion capacity of a water−soluble emulsifier is defined as the maximum amount of oil that can be dispersed in an aqueous solution containing a specific amount of the emulsifier, without the emulsion breakdown or inverting into a water−in−oil emulsion.

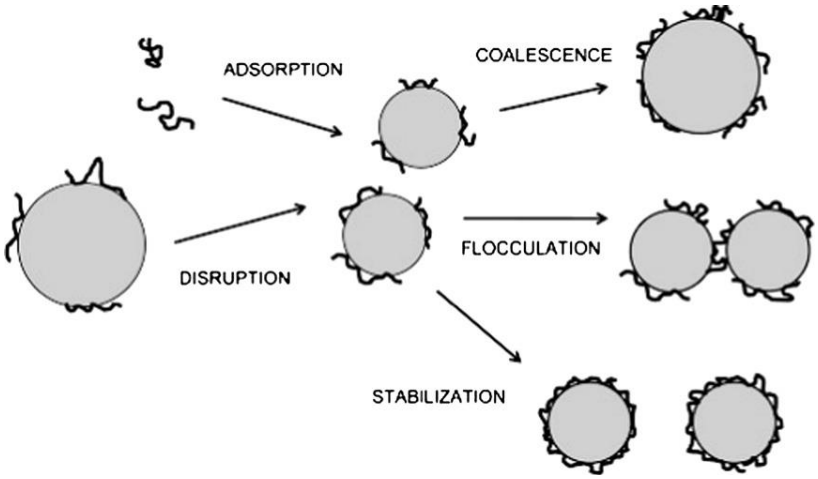
The problem of emulsions is that they are intrinsically unstable and with time the droplets of the dispersed phase will be attracted to each other and the emulsion will undergo changes which will result in the separation of oil and water phase. In o/w emulsions destabilization results in flocculation, coalescence, creaming and oiling−off, which may occur singly or in combination form, as shown in Figure 2.9. The main processes involved in the phenomenon of emulsion destabilization include:

a. Creaming and drainage: Because the density of two phases is different, the oil phases normally being less dense than the aqueous phase, leading to a movement of the dispersed phase.
b. Flocculation: it is normally a reversible loose aggregation of droplets held together by weak force. A flocculation will depend on the volume fraction and the strength of the interaction between the droplets.
c. Coalescence: If the attractive force is great enough to overcome the energy barrier to bring two droplets into close proximity with one another and the droplets will coalesce.
d. Phase inversion: Phase inversion occurs when an o/w emulsion become a w/o emulsion or w/o become o/w. It is complex and probably involves creaming, flocculation and coalescence. The volume fractions in the two phases are very important factors in the change from one phase to another.

The stability of an emulsion depends on the balance of the forces associated with the interface of the oil and water, which are attractive force, repulsive forces, steric force and depletion force. The stability of food emulsions is complex because it covers both a number of phenomena and a variety of systems with different forces (Dickinson, et al., 1991, 1992).



(A)



(B)

Figure 2.9. (A) Schematic representation of the various breakdown processes in emulsions (Tharwat & Tadros 2013) and (B) Illustration of main physico-chemical processes involved in making of emulsions. Stabilization of fine droplets requires mechanical disruption of coarse droplets accompanied by rapid effective adsorption of emulsifier at the new oil–water interface. Collision of droplets with insufficient coverage of emulsifier leads to coalescence and/or flocculation (adapted from Dickinson, 2008).

2.7.4 EMULSIONS STABILIZED WITH PROTEINS

2.7.4.1 General aspects of emulsions stabilized with proteins

Mixtures of milk proteins are widely used as ingredients in various food products because they are excellent emulsifiers (Dickinson, 1999). Homogenization is an essential step in emulsion formation, in which large oil droplets are broken down by the vigorous application of mechanical energy (Walstra & Jenness, 1984). During the course of emulsification, milk proteins are capable of rapidly adsorbing at the surface of the newly formed oil droplets, reducing the interfacial tension and forming thick layers which prevent droplets from coalescence or flocculation via steric and electrostatic stabilization mechanisms (Figure 2.9) (Dickinson, 2008; Tharwat & Tadros 2013).

Protein adsorption at the oil−water interface (Figure 2.9) is thermodynamically favourable because hydrophobic residues of the protein backbone are removed from the bulk aqueous phase and are oriented towards the oil phase, following protein structural rearrangement at the interface (Dickinson, et al., 1988). As a result, the adsorbed globular protein structure lies somewhere intermediate between the native state and the fully−denatured state, sometimes referred to as the molten globule state (Dickinson, 1998).

The behaviour of oil−in−water emulsions in foods is defined by the three parts of the system; the oil phase, the aqueous phase and the interfacial layer between the lipid material and the bulk phase. The stability of emulsions is correlated to a large extent to the interactions between the droplets, which in turn depend on the structure and composition of the adsorbed milk protein layers at the surface of the fat globules (Dalgleish, 2006). Nevertheless, despite the fact that the composition of a milk protein interfacial film can be adequately investigated, the structures of the adsorbed emulsifiers remain unknown with respect to their molecular detail. Furthermore, proteins at the interfacial layer of the emulsion droplets may be capable of interacting with the aqueous phase components. These types of interactions between proteins in adsorbed layers and proteins in solution depend on the chemical environment of the bulk phase. For instance, a general increase in ionic strength can destabilize the emulsion, which is attributed to a masking effect on the charged, adsorbed proteins

(Casanova & Dickinson, 1998).

2.7.4.2 Milk proteins at the oil–water interface

During homogenization of milk protein emulsions, competitive adsorption between the caseins and the whey proteins occurs, which results to the formation of a thin layer (~10 nm) consisting of both types of proteins (Millqvist−Fureby, et al., 2001). This maximum amount of protein adsorbed per surface area of the fat globule (2 – 3 mg / m^2) is assumed to be reflected by a monolayer of both caseins and serum proteins (Pelan, et al., 1997). As a general rule, the protein that arrives first at the interface is the one that predominates (Dickinson, 1997). Nevertheless, the ability of a protein to be the main species at an oil−water interface during or after emulsification depends mainly on its molecular flexibility and its surface hydrophobicity (Dickinson, et al., 1991). This seems to be the main reason for the preferential adsorption of the casein fraction in homogenized dairy emulsions (Tomas, et al., 1994). Caseins predominate at the oil−water interface because of their higher proportion of hydrophobic residues and the more flexible, open molecular structure compared to the whey proteins (Dalgleish, et al., 2002). The flexible, amphiphilic caseins adsorb fast during homogenization and lower the surface tension. This ability exhibited by caseins makes them the dominant species at the stabilizing layer. However, experimental evidence indicates that proteins will adsorb to the oil interfaces in proportion to their concentrations in the aqueous phase (Hunt & Dalgleish, 1994). This statement is further strengthened by the studies (Ye, 2008) which indicate that the interfacial composition of emulsions made with mixtures of sodium caseinate and whey protein concentrate depend on the protein concentration. Caseins adsorb preferentially at the oil−water interface at high protein concentrations, whereas at low protein concentrations (< 3 %), whey proteins adsorb in preference to caseins. Furthermore, once the milk proteins become adsorbed at the oil−water interface, little free reversible exchange occurs between the adsorbed proteins and the proteins located at the aqueous phase (Dickinson, et al., 1990). It has been documented (Dickinson & Matsamura, 1994) that when globular proteins such as β−lactoglobulin are adsorbed, displacement from the surface layer by other proteins, even very

flexible ones like β−casein or α_{s1}− casein, is rather unlikely to occur. It has been suggested however that whether one protein will displace another protein from the oil interface depends not only on their relative molecular flexibilities, but also on the density of interfacial packing and the strength of interfacial protein−protein interactions for the species forming the layer covering the oil droplet (Dickinson, et al., 1988).

According to previous studies, α−La may be displaced from the interface by β−casein, due to the inability of the whey protein to polymerize via sulfhydryl exchange reactions (Dickinson, 1989).

MPC can act as emulsifiers and emulsion stabilizers to provide an absorbed layer at the oil / water interface as it is being used in food emulsions and food products. The interfacial properties (the thickness, cohesiveness and charge of layer formed by the adsorbed protein) are important in emulsification. When emulsion is formed, the emulsion qualities are influenced by:

- Interfacial tension between the two phases
- Characteristics of the adsorbed film in the interface
- Magnitude of the electrical charge on the fat globules
- Size and surface / volume ratio of the globules
- Weight / volume ratio of dispersed and dispersion phases
- Viscosity of the dispersion phase.

The emulsification properties of experimentally controllable parameters are:

- Type of equipment used to produce the emulsion
- The energy input into the system
- Amount of protein used
- Phase volume used
- Ionic strength
- pH
- Type of oil used.

The emulsifying properties of MPC and the stability of the emulsions formed with MPC were influenced by the casein micelle size of casein micelles induced by reduction of Ca content in the MPCs were demonstrated by Ye (2011). They found that when casein micelles in the MPCs dissociated because of a

reduction in the Ca content, the emulsifying ability was improved through the formation of a fine emulsion with smaller droplet size at a lower protein concentration. The fine emulsions demonstrated a high stability at low surface concentration. Sikand, et al. (2012) also reported that the process of MPC production affects the mineral composition, protein concentration and structure, which in turn influenced their functionality. And change in temperature, pH and ionic strength before and during milk protein concentration was shown to have influence on the alteration of casein micelle size, in turn the solubility, heat stability and emulsion properties.

Chapter 3. **Experimental Techniques**

This chapter has described the experimental design, procedures for sample preparation, methods used for the physico−chemical characterisation and functionalities of the samples.

3.1 Experimental Design for Raw Milk

To reflect industry situation and to avoid any influence of pre-treatment (e.g. pasteurisation) to the milk, raw milk was investigated with regard to the change in temperature, pH and the addition of chelators, individually or in combination, on the casein micelle size and zeta potential of casein micelle and soluble and ionic Ca.

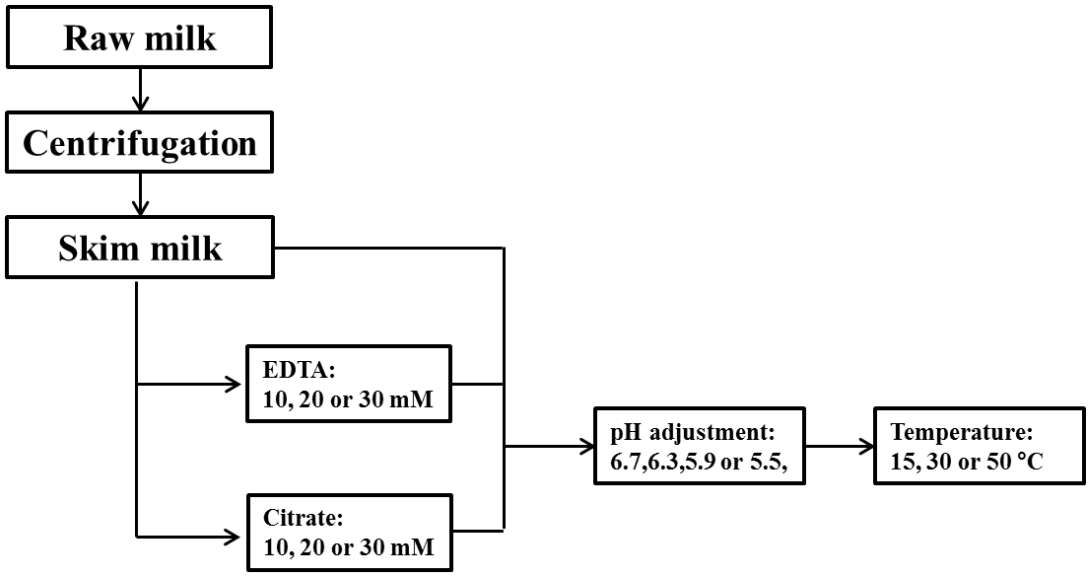


Figure 3.1. Flow diagram for raw milk sample preparation.

In this study, the fresh raw milk from local farm, defatted in a laboratory scale, was used and the effect of temperature, pH and addition of Ca chelators on soluble and ionic Ca and the casein micelles size was investigated (Figure 3.1).

3.2 Experimental Design for UF of Skim Milk

In this part, the pasteurised skim milk was purchased from local supermarket (Woolworths, Melbourne, Australia), stored at 4 °C and was used within two or three days. The skim milk composition and nutrition value were shown in Table 3.1.

Table 3.1. Composition and nutrition information of the skim milk.

Milk Composition		**Per 100ml**
Protein		3.4 g
Fat -	−Total	0.1 g
	−Saturated	0.1g
	−Trans	0.0 g
	−Polyunsaturated	0.0 g
	−Monounsaturated	0.0 g
Carbohydrate		5.3 g
Sodium		40 mg
Calcium		120 mg

The skim milk was subjected to UF using membrane at 15, 30 or 50 °C to produce MPC, or adjusted to pH 6.7 (control), 6.3, 59 or 5.5; or added with Ca chelators (EDTA or citrate at 10, 20 or 30 mM) prior to UF. The retentates, the liquid form of MPC, were freeze dried to obtain dried MPC powders. The physicochemical characteristics of the retentates and the functionalities of the reconstituted MPC were analysed using the methods described below. The overall experiment design is shown in the following diagram (Figure 3.2).

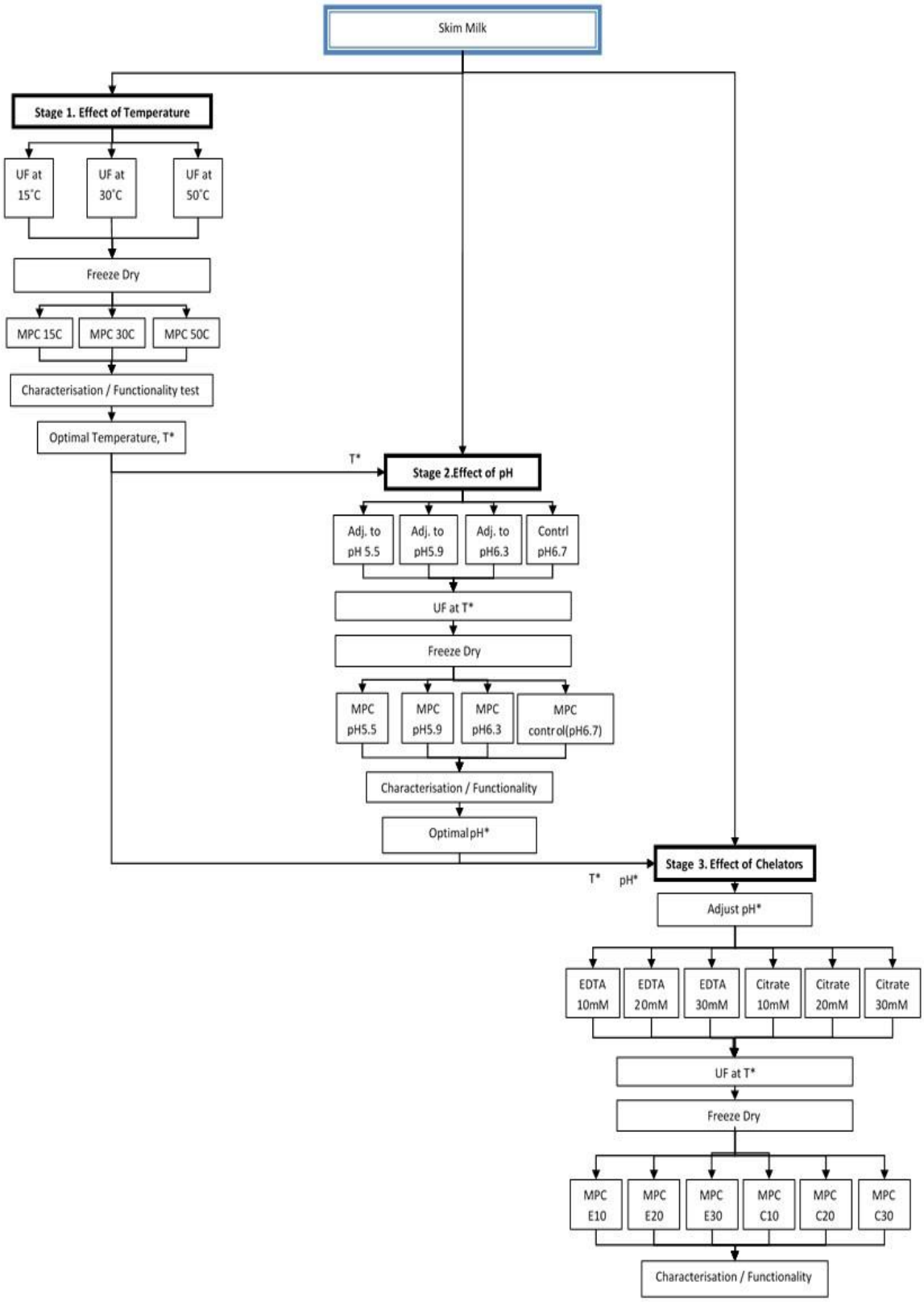


Figure 3.2. Outline of project flow diagram and the dependence between stages.

3.2.1 INVESTIGATION OF THE EFFECT OF OPERATION TEMPERATURE ON THE UF PROCESS AND THE FUNCTIONALITY OF MPC

The effect of UF operation temperature (T = 15, 30 or 50 °C) on the membrane performance and MPC functionality was investigated. Skim milk was subjected to the UF using membrane at temperatures of 10, 30 or 50 ˚C, respectively. The permeate flux was recorded every 30 min. Total solids, casein micelle size, zeta potential, protein, and FTIR analysis of the retentate were conducted at the start, in the middle and at the end of UF process. FTIR was used to investigate whether there is protein conformational change during the UF process. The membrane after use, when the retentate reaches five times concentration, was examined using scanning electron microscopy (SEM). The feed skim milk and retentate collected during UF process and at the end of filtration were freeze dried. Part of the retentate and permeate were analysed for total, ionic and soluble Ca content. The freeze dried retentate, which is known as MPC, was characterised for functionalities, e.g. solubility, heat stability, emulsion capability and stability. Based on the membrane performance and MPC functionality, an optimal temperature, T*, was determined for use next.

3.2.2 INVESTIGATION OF THE EFFECT OF pH OF THE SKIM MILK ON THE UF PERFORMANCE AND FUNCTIONALITY OF MPC

The skim milk was adjusted to pH of 5.5, 5.9 or 6.3 prior to UF using 1 M or 0.1 M HCl or NaOH. The pH adjusted skim milk together with the natural skim milk (pH 6.7) were UF'ed operated at the selected temperature, T*, determined in the previous experiment. Membrane performance and MPC characterisation were carried out similar to that in the last experiment. Based on the membrane performance and MPC functionality, an optimal pH* was selected for use next.

3.2.3 INVESTIGATION OF THE EFFECT OF CHELATORS ON THE UF MEMBRANE PERFORMANCE AND THE FUNCTIONALITIES OF MPC

Skim milk was adjusted to the pH* (optimal pH determined in the last experiment) and added with 10, 20, or 30 mM EDTA, or 10, 20 or 30 mM citrate, and then UF'ed at T* determined in previous experiment. The

membrane performance and MPC characterisation were carried out similar to previous experiment.

3.2.4 MEMBRANE SYSTEM

A lab scale membrane system consists of a feed / retentate tank, feed pump, plate membrane cell, permeate tank and a balance as shown in Figure 3.3 and Figure 3.4.

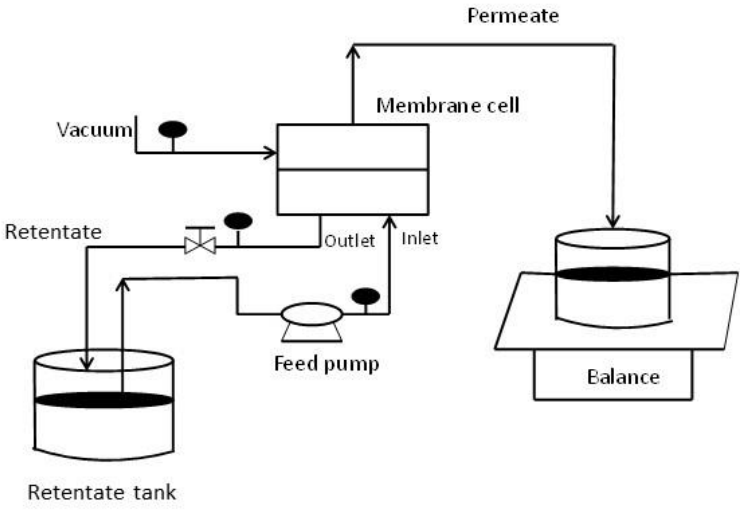


Figure 3.3. Schematic diagram of membrane UF set up.

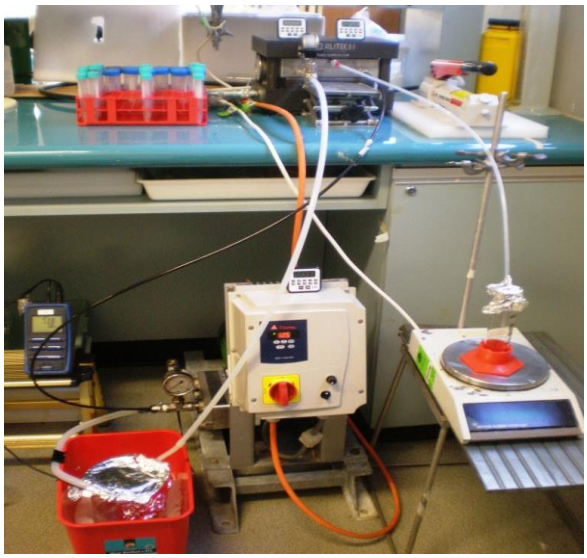
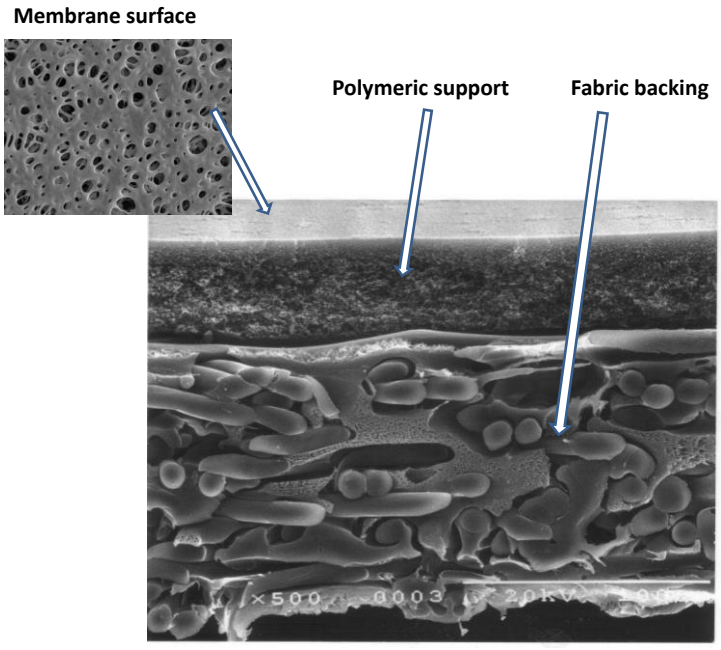


Figure 3.4. Photograph of the laboratory membrane UF set up.

Membrane modal and PES membranes (Figure 3.5) both manufactured by

SterliTech Corporation (Kent, WA, USA) and Microdyn−Nadir GmbH (Wiesbaden, Germany) with membrane molecular weight cut−offs (MWCO) of 20 kDa, was used. The membranes were supplied as flat sheets and were stored dry under ambient conditions.

(A)



(B)

Figure 3.5. (A) Photograph of a SteriTech membrane module and (B) surface and cross section of PES membrane microstructure.

3.2.5 PREPARATION OF MILK CONCENTRATES

A membrane module (SEPA CF) and polyethersulfone (PES) membrane (190 x 140 mm) with a molecular cut−off of 20 KDa were both purchased from SterliTech Corporation (Kent, WA, USA) and used for the UF.

The set up was conditioned to the temperature of operation by running Milli−Q water at the same processing temperatures before running the milk sample.

The steps involved in treatment of the skim milk, in its provided state as a control, or pre−treated in accordance to the corresponding experimental requirements (temperature, pH, Ca chelators) were described in each following chapters. The milk used for this study was skim milk commercially pasteurized (72°C / 15 s) provided by a local supplier (Woolworths, Melbourne, Australia). The sodium azide ≥ 99 % (Product number: 13412; Sigma−Aldrich) in concentration of 0.02 % was added into milk for sample preservation.

In each experiment, the skim milk was pumped through the membrane module at a pressure of 2 bar using a hydra−cell pump (T−VERTER 220V 1.5 KW 2HP, Wanner Engineering Inc., Minneapolis, MN, USA) and concentrate (retentate) was recycled into the feed container until the volume was reduced to 1 / 5 of its original volume (VCF of 5). This was monitored by measuring the weight of permeates during UF. Samples of permeate and retentate, collected at time 0 (at start), 2 and 4 h, and at the end of operation, were analysed immediately for casein micelle size, zeta potential and conformational changes of proteins using an FTIR. Part of the concentrated samples was stored at −20 °C and used for Ca and protein analyses, and the remaining part of the concentrates was freeze dried (Dynavac, Model FD300, Dynavac Eng. Pty. Ltd., Melbourne, Australia) and, later, reconstituted (5% TS) for the functionality (e.g. solubility, emulsion, etc) analysis. New membrane sheets were used for each UF run, which were wetted with Milli−Q water over night before use.

3.2.6 DETERMINATION OF UF OPERATING CONDITION

It is known that permeate flux increased with an increase of feed rate and inlet pressure; this was the common situation for liquids with less solids (Angela, et al., 2013). However in the case of the milk, some of the proteins would be left

on the membrane surface. An increase in flow rate and pressure level would induce an inside effect on the membrane fouling; therefore, a preliminary experiment was conducted to determine the optimal operation conditions (flow rate and cross membrane pressure). The criterion used to determine the optimal operating condition was the maximum permeate flux or the minimum time required to reach 5 times concentration of skim milk at room temperature. A feed flow rate at pump speed of 12.5 Hz and 2 bars were found to be the optimum operation condition for the native skim milk at room temperature (20 − 25 °C) therefore these flow rate and cross membrane pressure were used for all the sample preparations.

3.3 Sample Analyses

Figure 3.6 summarised the analyses and methods used for the experiments.

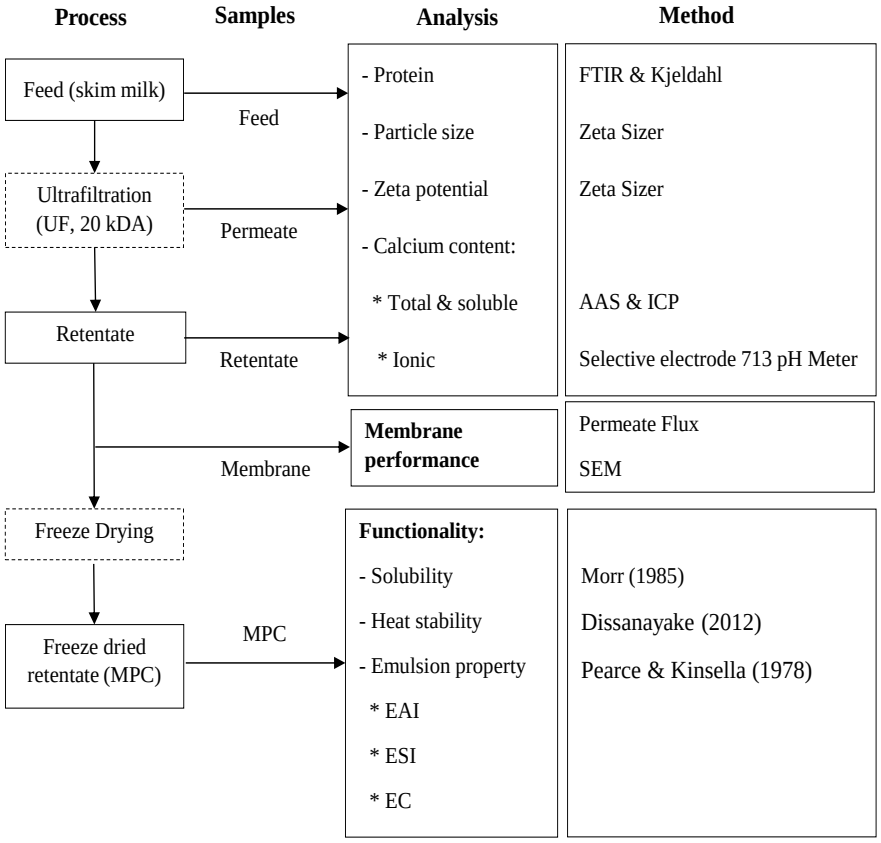


Figure 3.6. Analysis and methods. Note, EAI (emulsion activity index); ESI (emulsion stability index); EC (emulsion capacity).

3.3.1 PHYSICAL PROPERTIES

3.3.1.1 Total solids

Total solids were determined by dry−weight analysis, using 10 ml moisture dishes with a milk sample placed in the oven at 105 °C for overnight. The samples collected at time 0, 1, 2, 4 hrs and at end of UF were mixed well before placing into the oven, and left at the desiccator for about 30 minutes, then weigh and calculated to obtain total solids value.

3.3.1.2 Casein micelle size and zeta potential

Casein micelle size and zeta potential of the skim milk, retentate and permeate samples were measured immediately after the collection of samples using Malvern Zetasizer (Model ZEN 3600, Malvern Instruments Ltd, Worcestershire, UK). The samples were diluted with Milli-Q water as required (1: 100 for 0 h milk samples and 1: 1000 for concentrated retentate samples) to meet quality data requirement during measurement (Melema, et al., 2009). Upon dilution, the samples were vortexed for 10 − 15 s and immediately introduced into the instrument. All measurements were carried out at room temperature (22 °C). Each measurement took about 10 min to complete, which limited mineral equilibration between the casein micelles and aqueous environment and thus maintained the integrity of the micelles, as is required for an accurate measurement (Beliciu & Moraru, 2009). Triplicate measurements were conducted on each sample. The refractive index and viscosity of water used in calculations were 1.330 and 1.1442 m.Pa.s, respectively. The applied voltage was set at 40 V and the dielectric constant was 82.2. Hydrodynamic diameter (D_h) was measured by dynamic light scattering (DLS) in the same instrument under the same conditions using a zetasizer software. Measurements were carried out at a scattering angle of 173° and wavelength of 633 nm. The average D_h was calculated using the Stokes−Einstein relation under assumption that particles had a spherical shape (Silva, et al., 2013). The results are presented as a volume distribution. The experimental error on the average D_h was 5 nm.

3.3.2 CHEMICAL PROPERTIES

3.3.2.1 Calcium content

Total Ca

The total Ca content of the permeate and retentate was determined using an Atomic Absorption Spectrophotometer (AAS) (AA−6300 SHIMADZU Shimadzu Corporation, Kyoto, Japan) at 422.7 nm wavelength following the AOAC official method of analysis (AOAC, 2005) with some modifications on the sample preparation as suggested by ST−Gelais (1992). The samples were prepared using 15 % (w/v) trichloroacetic acid (TCA) and 10 % (w/v) lanthanum chloride (Sigma−Aldrich Pty Ltd, Castle Hill, NSW, Australia) and suitably diluted in order to comply with a range of standard concentrations using Milli−Q water. The Ca content was calculated and expressed as mM.

The Ca retained on the membrane was also measured by analysing the Ca content of the membrane ash. Ash was obtained by taking two pieces of the membrane, one from the edge and the other from the centre of the exposed area (Delaunay, et al., 2006) and following the standard AOAC protocol (AOAC, 2005). The Ca content was expressed in µg / cm^2 (Hausmann, et al., 2013).

Soluble Ca

To measure the soluble Ca, the sample was placed in centrifuge tube and centrifuged at 33,000 g for 1 h at room temperature (~20 ± 2 °C). The supernatant was carefully collected without disturbing the pellet at the bottom of the tube. A 1 ml supernatant was diluted to 10 ml first. Then 3 ml was taken from diluted samples with addition of 1 ml of TCA (for precipitate protein from the samples) and 1 ml of $LaCl_3$ (isolate from Mg and other metals) and diluted to 15 ml, which was kept still for 15 mins at 38 °C, then centrifuged at 4000 rpm for 10 min at room temperature; and the final clean supernatant collected for soluble Ca determination. A further dilutions was required to obtain accurate measurement so the concentration was within the range of 1 − 10 ppm standards, e.g. for 0 h sample, a dilution factor of 5x was required and for final retentate sample, 10x was required.

Ionic Ca

Ionic Ca concentration ($C_{Ca^{2+}}$) was measured of 5 % dispersion from 5 time retentate and original permeates using Ca ionic selective electrode connected to pH−713 lab meter (CT−06907−0047, Omega Engineering Stanford) fitted with a reference Ag / AgCl electrode. Calibrations were carried out using a range of $CaCl_2$ solution from 0.0003 to 0.025 M with an ionic strength of 0.08 M adjusted to KCl. A standard curve of potential vs $\ln(a_{Ca^{2+}})$ was used. The Ca^{2+} activity ($a_{Ca^{2+}}$) of this standard solution was calculated as:

$$a_{Ca^{2+}} = \frac{Y_{Ca^{2+}} C_{Ca^{2+}}}{C^0}$$

Where C^0 is the concentration of the chosen standard state, 1 mM / L, and Yca^{2+} = 0.425 and 0.403 are the activity coefficient of the Ca^{2+} as given by Debye − Huckl approximation (MacInnes, 1961) at 25 °C and 50 °C, respectively. The calibrations were performed every time prior to measurements of each batch. The standard curve was reproducible. The max temperature of the electrode was suggested to be ≤ 60 °C although it was stated the upper limit was 80 °C by the manufacturer (Chandrapala, et al., 2010a). This electrode temperature was safe for this study as the maximum temperature of milk sample was 50 °C. Then the Ca activity Ca^{2+} was calculated back to Ca concentration ($C_{Ca^{2+}}$) according to the standard measurements with the R^2 value = 0.99.

Determination of Ca content on the surface of the used membrane

The determination of the total Ca and soluble Ca from membrane followed the protocol described by ST−Galais, et al. (1992). The samples were first ashed at 550 °C overnight and then the ash was solubilized in 0.1 M HCl and filtered through Whatman No. 45 papers. The total and soluble Ca were analysed using the method described above.

3.3.2.2 Protein content

Kjeldahl method

The total protein, casein nitrogen, non-protein nitrogen were measured using Kjeldahl methods based on the Association of Official Analysis Chemicals (AOAC) procedure. An N to protein conversion factor of 6.38 was applied.

- *Total protein*

The total protein nitrogen was measured using the Kjeldahl method as described by AOAC (2005). The protein conversion factor of 6.38 was applied.

Briefly, 1 g sample was taken and placed in a Kjeldahl digestion tube. Using forceps, 2 catalyst tablets (Kjeldahl Cu 3, 5) and sulphuric acid (12.5 ml) were added to each digestion tubes. Digestions took about 45 mins. Boric acid solution was used to collect the distillate. 0.1 M HCl was used for the titration of distilled samples. The following equation was used to calculate the nitrogen and crude protein content of milk samples:

$$Protein\ Content = (Nitrogen\ Content, \%\ N) \times (Protein\ Conversion\ Factot, F)$$

Protein analysis of the samples was conducted using Kjeldahl method with a nitrogen conversion factor of *F* = 6.38 (AOAC, 2000; method 968.06).

- *Casein nitrogen*

The original sample (retentate) of 2 ml was adjusted to pH 4.6 using 1 M HCl and kept for 10 mins to settle and then kept in water bath at 30 − 35 °C for 30 mins. The samples were centrifuged at 4000 rpm at 30 − 35 °C for about 10 mins. The pellets were weighed and diluted to 4 ml. Then, 1 ml of the diluted sample was taken to measure the casein nitrogen content by following the same procedure as that of total nitrogen (AOAC, 2000; method 968.06).

- *Non-protein nitrogen*

A 15 % of trichloroacetic acid (TCA) solution was firstly prepared. Then the milk samples were warmed to about 38 ˚C and mixed thoroughly. The milk was mixed with 4 parts of 15 % TCA solution and allowed for precipitate (casein micelle) to settle for 5 min. The solution was filtered using Whatman No.1 paper

(15 cm diameter, N−free) and the entire filtrate was collected. The filtrate should be clear and free of particulate matter. A 20 ml of filtrate was added into the Kjeldahl digestion tubes and followed the standard Kledahl method. The blank solution should contain about 15 % TCA and no sample. The determination of non−protein nitrogen follows the same procedure as in the method of total nitrogen in milk−Kjeldahl methods.

Bioanalyzer

The protein profile of the feed and corresponding retentates obtained at different time points was also characterised using a High Sensitive Protein 250 K Bioanalyzer 2100 (Agilent Technologies GmbH, Waldbronn, Germany). The samples were prepared and analysed under non−reducing conditions as per the instructions of the manufacturer. The samples were introduced into an Agilent High Sensitivity Protein 250 kit, which contains a chip and reagents for labelling of proteins. The assay consists of two major analytical steps involving covalent labelling of proteins with a fluorescent dye (blue) and separation and detection of labelled proteins with on−chip−electrophoresis (Agilent). Additionally, commercial samples of whey protein isolate (WPI, BiPro, Davisco International Inc, Eden Prairie, MN, USA) and sodium caseinate (NaCN, NatraPro, MG Nutritionals, Murray Goulburn Co−operative Co. Ltd., Melbourne, Australia) were used as references. The resolved peaks were compared with the external ladder protein standard, which was part of the kit.

3.3.2.3 ATR-FTIR

The change in protein conformation was observed using a Shimadzu IRAffinity−1, Attenuated Total Reflectance−Fourier Transform Infrared (ATR−FTIR) Spectrofluorometer (IRAffinity-1, Shimadzu Corporation, Kyoto, Japan) equipped with IRsolution FTIR software (Shimadzu). The spectra of the milk and corresponding retentates were obtained in the absorbance mode using Vee−Max flat plate ZnSe ATR crystal (45 degrees; PIKE Technologies, Inc., WI, U.S.A.). To measure the change on the protein conformation, the instrument was turned on 10 mints before sample was collected. The special sample preparation was no needed. The feed and retentate samples before and after UF were measured immediately after collected. Three samples in each

condition were measured twice. For each spectrum, an average of 40 scans was recorded at 4 cm^{-1} resolution in the range of 400 − 4000 cm^{-1} after atmospheric background subtraction. The interferograms thus obtained were ATR corrected, baseline adjusted to zero and smoothed (10 points) with the aid of the software in order to recognize the corresponding peaks under the broad Amide I region of 1600 − 1700 cm^{-1}. The peak areas obtained from peak tables of the manipulated spectra were used to identify and monitor changes in the FTIR spectra of the samples.

The use of Fourier transform infrared spectroscopy (FTIR) to determine the secondary structure of biological macromolecules in protein has dramatically expanded (Curley, et al., 1998). FTIR is routinely used by laboratories specializing in milk analysis because it is a fast, non−destructive, and easy procedure that enable simultaneous measurement of several components in a complex natural media (Griffiths & de Haseth, 1986; Etzion, et al., 2004; & Andersen, et al., 2002). It requires only small amount of proteins (1 mM) in variant environments, therefore, high quality spectra can be obtained without problems of background fluorescence, light scattering and problems related with the size of the protein.

The determination of protein conformation is based on the characteristic absorbance of milk protein of absorbance bands in the 1500 − 1700 cm^{-1} range known as the amide I and amide II bands. The absorption associated with the Amide I band leads to stretching vibrations of the C = O bond of the amide, and the absorption associated with the Amide II band leads primarily to bending vibrations of the N—H bond. Because both the C = O and the N—H bonds are involved in the hydrogen bonding that takes place between the different elements of secondary structure, the locations of both the Amide I and Amide II bands are sensitive to the secondary structure of a protein. Studies with proteins of known structure have been used to correlate systematically the shape of the Amide I band to secondary structure (Byler & Susi 1986; Surewicz & Mantsch, 1988). The Amide II band, though sensitive to secondary structure, is not as good a predictor for quantitating the secondary structure of proteins.

3.4 Determination of Membrane Performance

New polyethersulfone membrane sheets were weighed before use and then wetted with Milli−Q water at room temperature overnight and weighed again in order to test the amount of water absorbed by the membrane. A new membrane sheet was used for each experiment. After use, the membrane was weighed, then rinsed gently with water, was allowed to dry at room temperature and kept in −20 °C freezers immediately after experiment. The performance of membrane was evaluated by measuring the permeate flux (every 30 min) and examining the membrane surface and its cross section using scanning electron microscope (SEM).

3.4.1 FLUX MEASUREMENT

Permeate flux was measured every 30 min, by weighing the permeate weight (g) per 60 seconds (1 min) in small beaker placed on the balance, and calculated as below.

$$The\ permeate\ flux = \frac{Weight\ of\ permeate\ (kg)}{Membrane\ active\ area\ (m^2) \times Time(h)}$$

The weight of the retentate was monitored continuously and also measured by collection of permeate to determine the weight reduction.

3.4.2 MEMBRANE MICROSTRUCTURE

The used membranes were rinsed with water, dried under room temperature and stored at −20 ˚C before SEM examination. The SEM examination of membrane surface and cross section was carried out using JCM 5000 bench top NeoScopy Scanning Electron Microscopy (Swanzey Internet Group, Manchester, UK). The sample for surface examination was cut from the middle of the membrane to a size of 2 x 2 mm. The cross section samples of the treated membranes were prepared by wetting the membrane in Milli−Q water followed by cutting it in liquid nitrogen to a size of around 2 x 3 mm. The membrane samples were mounted on double sided carbon tape, allowed to dry

at room temperature overnight and coated with gold to prevent static electricity charge during examination. The SEM was set at Vac−high PC−STD 15 KV during examination and imaging.

3.5 Determination of MPC Functionality

3.5.1 MPC POWDER PREPARTION

The liquid skim milk concentrates (retentates) after UF was collected and separated into small tubes (50 mL) to the half of the tube volume to prevent overflow during freeze drying. The separated concentrates were frozen in a −20 °C freezer (WCM5000WC Chest Freezer, Westinghouse, Australia) at least overnight to let sample fully frozen. The frozen samples were moved to the freeze dryer and open the lids and kept enough space from each other to avoid samples from mixing during the evaporation.

MPC powders were prepared from retentates by freeze drying (DYNAVAC, FD 300 Freeze dryer) for 72 h (Pre−freeze drying 30 s, primary drying 40 h and secondary drying 24 h) under vacuum pressure of 100 kPa and condenser temperature −20 °C. After freeze drying, the tubes were closed, placed in yellow top plastic container (250 mL) and kept in −20 °C freezers.

3.5.2 MPC FUNCTIONALITY

MPC dispersion of 5 % w/w powder in water was subjected to assessment of solubility, heat stability, emulsion capacity, emulsion activity and emulsion stability.

3.5.2.1 Solubility

A 5 % (w/w powder in water) dispersion was prepared from freeze dried MPC powders by mixing them with Milli−Q water followed by stirring for 2 h on a magnetic plate at room temperature (22 °C) and added 0.02 % NaAg to prevent the grow of common microbial. The dispersion was left at 4 °C overnight to ensure complete hydration of the MPC powders. The MPC powders contained between 52 to 55 % protein on a dry base.

Protein solubility was estimated using the method developed by Morr, et al. (1985) with some modifications. Briefly, an aliquot of 10 ml of MPC dispersion was centrifuged (Centrifuge Model J2HS, Beckman, Fullerton, CA, USA) at 3000 g for 25 min at 10 °C. The supernatants were assessed for their protein content by the Kjeldahl method. The solubility was estimated using the following equation:

$$Solubility\ (\%) = \frac{Protein\ content\ in\ supernatant}{Total\ protein\ content}$$

3.5.2.2 Heat stability

The dispersion preparation for the heat stability was the same as described in solubility test.

The heat stability of MPC was measured by the solubility method as described by Dissanayake, et al. (2011). Briefly, approximately 3 ml of the MPC dispersion was sealed in narrow glass tubes (10 mm i.d. x 120 mm long), and placed in a rocking oil bath (Ratek, SP802, Australia Scientific Pty Ltd, Kotara NSW) set at 140 °C for 10 s. After 10 s, the tubes were removed from the oil bath and cooled instantly in an ice bath (~ 0 °C) followed by centrifugation at 3000 g for 25 min at 10 °C. The supernatant was filtered through 0.45 µm filter. The stability of heated dispersions was determined by measuring the protein contents as described above for solubility method and expressed using the equation:

$$Heat\ stability\ (\%) = \frac{Protein\ content\ in\ supernatant}{Total\ protein\ content}$$

3.5.2.3 Emulsion properties

Dispersion preparation

A 0.1 % (w/w powder in water) dispersion was prepared from freeze dried MPC powders by mixing them with Milli−Q water followed by gentle stirring for 2 h on magnetic plate at room temperature, and added 0.02 % NaAg to prevent the grow of common microbial. The dispersion was left at 4 °C overnight to ensure complete hydration of the MPC powders.

Emulsions preparation

The emulsions were prepared by mixing one part of canola oil (purchase from local supermarket, Coles, Melbourne, Australia) with three parts of MPC dispersion (0.1 % w/w protein content). A coarse emulsion pre−mix was first prepared by homogenizing oil and MPC dispersion for 60 min using a laboratory mixer (R30, Fluco electric stirrer with a 4 blade mixing element, Fluco, Shanghai, China), and keeping the mixture in a water bath maintained at 50 °C for 20 min. Thereafter the final emulsion was prepared by two stages of homogenization using an Ultraturrax (Pro Science PRO 250, Pro Scientific Inc., and Oxford, CT, USA) at 10,000 rpm for the first stage (5 min) and at 15,000 rpm for the second stage (5 min). The emulsions thus formed were immediately cooled to 25 °C.

Emulsion activity index (EAI)

The Emulsion Activity Index (EAI) and emulsion stability were calculated using the method described by Pearce and Kinsella (1978) by measuring the turbidity and oil volume fraction of emulsion and calculated by:

$$Emulsion\ Activity\ Index\ (EAI) = \frac{2T}{\varphi C}$$

Where, T is turbidity; ϕ is oil volume fraction; C is the weight of protein per unit volume of aqueous phase before emulsion is formed. The turbidity was measured with a UV / Vis spectrophotometer (Libra S11 Biochrom, Cambridge

CB4OFJ England). Plastic cuvettes (category 1941) with a pathway of 1 cm were used. Measurements were carried out at ambient temperature using a wavelength of 500 nm. Samples of aliquots (1 ml) emulsions are diluted serially with (0.1 %) sodium dodecyl sulphate (SDS) solution to give final dilutions of 1 / 3000.

Emulsion stability index (ESI)

The Emulsion Stability Index (ESI) was analysed after the emulsion was stored at 4 °C for 24 h. briefly, aliquots of 1 ml of the emulsion was diluted serially with 0.1 g / 100 ml sodium dodecyl sulphate (SDS) solution to give final dilution from 1 / 100 to 1 / 3000 according to the concentration of samples. The absorbance of the diluted emulsion was determined using 1 cm path cuvette at wavelength of 500 nm using UV / Vis spectrophotometer. The method for preparation of standard solution was according to the standard 3.1 ml assay protocol and procedure (Sigma−Aldrich, Catalogue No B6916) to measure the absorbance. The turbidity of the emulsion was calculated by:

$$T = \frac{2.303A}{I}$$

Where, T is turbidity, A is absorbance at 500 nm and I is path length of cuvette. The emulsion stability index was calculated using the equation:

$$ESI = \frac{T.\Delta t}{\Delta T}$$

where, T is calculated turbidity value at 0 h; Δt is time interval (24 h) and ΔT is change in turbidity during the time period (24 h).

Emulsion capacity (EC)

Protein dispersions were prepared by dispersing 1% w/w MPC powder in distilled water by gentle stirring for 2 h at room temperature. A 500 g protein dispersion was prepared using a magnetic stirrer at a speed of 6 on the stirring dial without applying heat. The pH of the solution was adjusted to 7.0 using 0.1 M NaOH as required. Exactly 50 ml of pH adjusted protein dispersion was measured into a 50 l measuring cylinder and transferred into a beaker sitting beneath the ultraturrax generator. It was ensured that the ultraturrax generator,

multimeter probes and oil outlet tubes were resting in the protein dispersion prior to commencing the test. The oil (~21 °C) was pumped at a flow rate of 20 ml oil / min and the speed of the ultraturrax was controlled at 10,000 rpm.

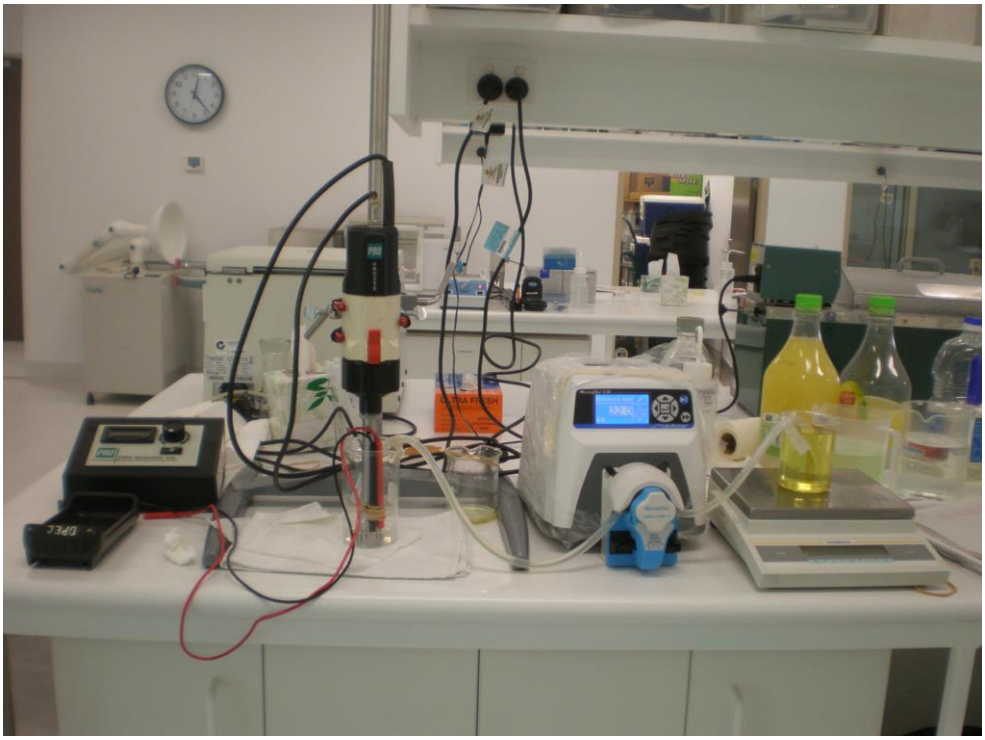


Figure 3.7. Laboratory set up for testing emulsion capacity.

The emulsion capacity (EC, g of oil / mg of protein) of the samples was determined by pumping oil into the MPC dispersion (~21 °C) with homogenisation while monitoring the electrical resistance of the dispersion (Figure 3.7). When the MPC dispersion exceeded its capacity to emulsify the added oil, the emulsion inverted from water in continuous phase to oil in continuous phase. This point was observed by a rapid increase in the electrical resistance of the system. This method was developed by Vuillemard, et al. (1990) and modified by Dairy Innovation Australia Ltd. (Werribee, VIC, Australia). The emulsion capacity was calculated using the equation:

$$EC = \frac{Weight\ of\ oil\ used\ in\ emulsion\ preparation}{Total\ protein\ content}$$

3.6 Statistical Analysis

All experiments were at least replicated with three sub-samplings ($n \geq 6$). One−way analysis of variance (ANOVA) at 95 % level of confidence was applied to analyse the differences caused by the main effectors. Tukey Honestly Significant Difference (HSD) post hoc test was applied to differentiate significant differences between the means at $P < 0.05$. The statistical analysis was performed using Advanced Analytics (SAS) software (SAS, 1996).

Chapter 4. **Effect of Temperature, pH and Ca Chelators on Raw Milk**

4.1 Introduction

Raw milk is the milk obtained directly from dairy farm without any pre-treatment. The milk components, in particular, the milk protein and minerals are not disrupted, remain in their native state in the raw milk.

In the dairy industry, pasteurization is a process used to keep milk from spoiling too quickly and safe to drink. However, pasteurization involves heating raw milk to high enough temperatures to kill off many microorganisms that can cause milk spoil. Such heat treatment also can induces some important changes to the milk, including a decrease in pH, precipitation of Ca phosphate, denaturation of whey proteins and interaction with casein, lactose isomerisation, modifications to the casein micelle and mineral partitioning. An overview of the changes taking place when milk is heated was given by Walstra & Jenness (1984). The extent of whey protein denaturation and alteration to the casein micelles is directly related to the severity of the heat treatment (Martin, et al., 2007). Because of heat treatment, some permanent changes to milk proteins (whey protein and casein micelles, etc.) and mineral partitioning may occur in the milk. The heat induced change on the skim milk has been studied for decades (Corredig & Dalgleish, 1999; Donato, et al., 2007; Raikos, 2010), however, information on effect of heating raw milk on partitioning of minerals and casein micelles in the raw milk was limited.

The milk protein denaturation and aggregation reported is affected not only by heating conditions but also by the milk environment, such as pH and ionic strength (Anema & Li, 2003b). The effect of temperature and pH on skim milk has been extensively studied (Walstra & Jenness, 1984; Nagy, et al., 2012; Liu & Guo, 2008; Vasbinder, et al., 2003). It is also known that Ca chelators can induce a shift of minerals between serum and micelles in the milk (Gaucheron, 2005; Post, et al., 2012). However, the effect of these parameters individually or

in combination on the change of raw milk properties is scarce.

In order to build a better understanding in the effect of temperature, pH and Ca chelators on milk constituents, we selected raw milk for this study before subjecting the milk to processing conditions to produce MPC, so as to avoid the influences from any pre-treatment. In this chapter, the fresh raw milk from local farm, skimmed in the laboratory, was used and the effect of temperature (15, 30 and 50 °C), pH (6.7, 6.3, 5.9 and 5.5) and addition of chelators (EDTA or citrate at levels of 10, 20 and 30 mM) on casein micelle size, zeta potential, soluble and ionic Ca in the raw skim milk was investigated.

4.2 Materials and Method

4.2.1 MILK PREPARATION

Raw milk collected from Warrnambool (Victoria, Australia) was heated to 50 °C in the water bath for 30 min and then skimmed using a cream separator (50 L/h, Milk Cream Separator, Kiev, NA, Ukraine) on the same day as described in section 3.1.4 (Figure 3.3). Then Na Azide (0.02 %) was added to the milk and kept in cool room (0 °C) overnight for subsequent treatment and analysis.

4.2.2 SAMPLE PREPARATION

The raw skim milk was taken from the cool room and placed in room environment until the temperature (21 ± 0.5 °C) was stable, then Ca chelators of EDTA or citrate was added (Figure 3.2). The samples with addition of 10, 20 or 30 mM of EDTA (coded as E10, E20 and E30) or citrate (coded as C10, C20 and C30) were prepared using either EDTA (200 mM $C_{10}H_{12}N_2Na_4O_8$. $2H_2O$, MW = 372.3) or tri−sodium citrate (200 mM $Na_3C_6H_5O_7$. $2H_2O$, MW = 294.10) as shown in Figure 4.1. Each chelator treated milk samples were divided into four groups and pH was adjusted to 6.3, 5.9 or 5.5 by slowly adding 1 M or 0.1 M HCl or NaOH with continuous stirring in order to prevent milk aggregation. The samples were then placed in a water bath at 15, 30 or 50 °C for about 30 min until the temperature was stable before sampling and analysis was carried out. The ionic Ca, casein micelle size and zeta potential measurement was

carried out immediately. The total and soluble Ca analysis was conducted from frozen milk samples. All analysis were carried out in triplicate and all the experiments were independently repeated (n = 6).

4.2.3 CASEIN MICELLE SIZE AND ZETA POTENTIAL MEASUREMENT

The casein micelle size and zeta potential of samples were measured immediately using Malvern Zetasizer (Model ZEN3600, Malvern Instruments Ltd, Worcestershire, UK). The samples were diluted 1: 100 using Mili-Q water to meet quality data requirement during measurement. Triplicated measurements as described in section of 3.3.1 were conducted on each sample and data was analysed using Malvern software.

4.2.4 CALCIUM CONTENT DETERMINATION

Total Ca and soluble Ca were determined using the methods described in section of 3.3.2.1. The samples were prepared using 100 % (w/v) trichloroacetic acid (TCA) and 10 % lanthanum chloride (Sigma−Aldrich Pty Ltd, Castle Hill, NSW, Australia) and suitably diluted using Milli−Q water. The Ca content was calculated and expressed as mM.

4.2.5 IONIC Ca DETERMINATION

The ionic Ca content was determined using Ca ion selective electrode connected to pH−713 lab meter (Omega engineering Stanford (CT−06907−0047) for Ca Activity) and calculated back to Ca concentration according to the standard curve. The calibration curve plotted the electric potential (mV) as a function of calculated logarithm Ca activity. The obtained Ca activity for each sample was converted into free Ca concentration using the Davies equation accounting to the Ca ion activity coefficient (γ = 0.425), at room temperature for the milk (Walstra & Jenness, 1984). The general relationship between activity a_i and concentration m_i is written as:

$$a_i = m_i . \gamma$$

where, γ is the molar activity coefficient, a_i is the Ca activity, and m_i is the

concentration of ionic Ca. R^2 value = 0.99.

4.2.6 STATISTICAL ANALYSIS

The experiment was replicated with three sub-samplings (n = 6). The original raw data of particle was analysis using Advanced Analytics (SAS) techniques. Other data were analysed using 1−way analysis of variance at 95 % level of confidence.

4.3 Results and Discussions

4.3.1 EFFECT OF CHELATORS ON THE COLOUR

Milk is a white or yellow−white complex fluid. Caseins are the major component found in bovine milk accounting for nearly 70 − 80 % of total protein in combination with Ca phosphate forming the casein micelles. This suspended micelle in the milk is mainly responsible for the white colour of milk. The loss of white colour was observed when EDTA and citrate (≥ 20 mM) was added to the raw skim milk (Figure 4.1). This suggested that the casein micelles structure has been disrupted by the addition of EDTA or citrate.

Milk with addition of EDTA **Milk with addition of citrate**

Figure 4.1. Photographs of raw skim milk after addition of EDTA (left: E10, E20, E30) or citrate (right: C10, C20, C30) at levels of 10, 20 or 30 mM (left to right).

In the serum phase of milk, salts exist either as free ions or as ion pairs / complexes. The ion equilibria is known to play an important role in the structure and stability of casein micelles (Horne, 1998; Walstra, 1990). Alterations of the ion equilibria induce significant changes in the ionic composition between the colloidal phase and aqueous phase. This will have effects on the physicochemical properties of casein micelles and their stability (De La Fuente, 1998; Fox & McSweeney, 1998; Huppertz & Fox, 2006). The loss of white colour is attributed to the disruption of casein micelle structure when CCP was dissolved by the addition of citrate or EDTA. This is in agreement with O'Mahony & Fox (2013), who observed that the white colour of skim milk was lost when citrate, EDTA or oxalate was added to skim milk.

4.3.2 EFFECT OF TEMPERATURE, pH AND CHELATORS ON THE CASEIN MICELLE SIZE

Average casein micelle size of the raw skim milk and that added with chelators (EDTA or citrate, 10, 20 or 30 mM) and adjusted to pH of 6.7, 6.3, 5.9 or 5.5 at temperature of 15, 30 or 50 °C is shown in Figure 4.2.

The average casein micelle size of the raw skim milk without addition of chelators was around 100 nm at 15 °C to 50 °C at natural pH of skim milk (pH 6.7). At pH 5.5, the casein micelle size was the same as that at pH 6.7 at 15 °C, which became noticeably larger at 50 °C (Figure 4.2). The casein micelle size was reported to be in the range between 176 − 177 nm at 20 °C, 200 nm at 40 °C and 194 nm at 50 °C (Beliciu & Moraru, 2009). Our observations differed since the average casein micelle size was half of that reported (Figure 4.2 A−C). Singh & Creamer (1992) found that casein micelle size increases in heat treated milk due to the attachment of denatured whey proteins to the casein micelle. In the current study, the temperature was kept below 50 °C, which was well below the whey denaturation temperature (about ~70°C) (Wijayanti, et al., 2014). The observed small casein micelle size in raw skim milk may either be due to the change in the density (Martin, et al., 2007), or because the serum casein micelles were present as small sub micelles at low temperature (Walstra, 1999), or due to the presence of endogenous proteases in the raw milk that may digest the micelles in this study (Luo, et al., 2015).

The casein micelle size reduction upon cooling of skim milk was also observed by Rose (1968), Downey and Murphy (1970), and Creamer et al. (1977), who attributed this to the dissociation and solubilisation of β−casein and other caseins from the micelles due to the weakening of the strength of the hydrophobic interaction between β−casein molecules and other caseins, which acted as integral components of the casein micelle structure (Swaisgood, 2003).

Addition of chelators (30 mM EDTA, or 20 and 30 mM citrate) into the raw skim milk increased casein micelle size of most samples (Figure 4.2). Among the samples tested, the addition of citrate was more effective in increasing casein micelle size than EDTA, low pH (e.g. 5.5) was more effective than high pH. Similarly, casein micelle size increases were greater at low temperatures than at high temperatures. The increase in casein micelle size upon addition of chelators to the skim milk is in strong contrast to the observation of other researchers (Udabage, et al. 2000, 2001) that casein micelle size decreased upon addition of 20 mM EDTA to commercial skim milk. This suggests that there is a difference in behaviour of raw skim milk and commercial skim milk. It is known that Ca chelator shifts the casein-mineral equilibrium, and causes dissolution of CCP from micelle and release specific caseins from the micelle (Holt, 1992; Gaucheron, 2005; Panouille, et al., 2004). Casein micelles may eventually dissociate into smaller clusters upon addition of chelators (Panouille, et al., 2004; Marchin, et al., 2007; Griffin, et al., 1988; Udabage, et al., 2000). However, the increase in casein micelle size observed upon addition of chelators to the raw skim milk may be because dissociated caseins and whey protein could have formed new protein clusters with larger sizes. This may also be the reason for the recovery of the white colour in the raw skim milk with the addition of 30 mM EDTA (Figure 4.1).

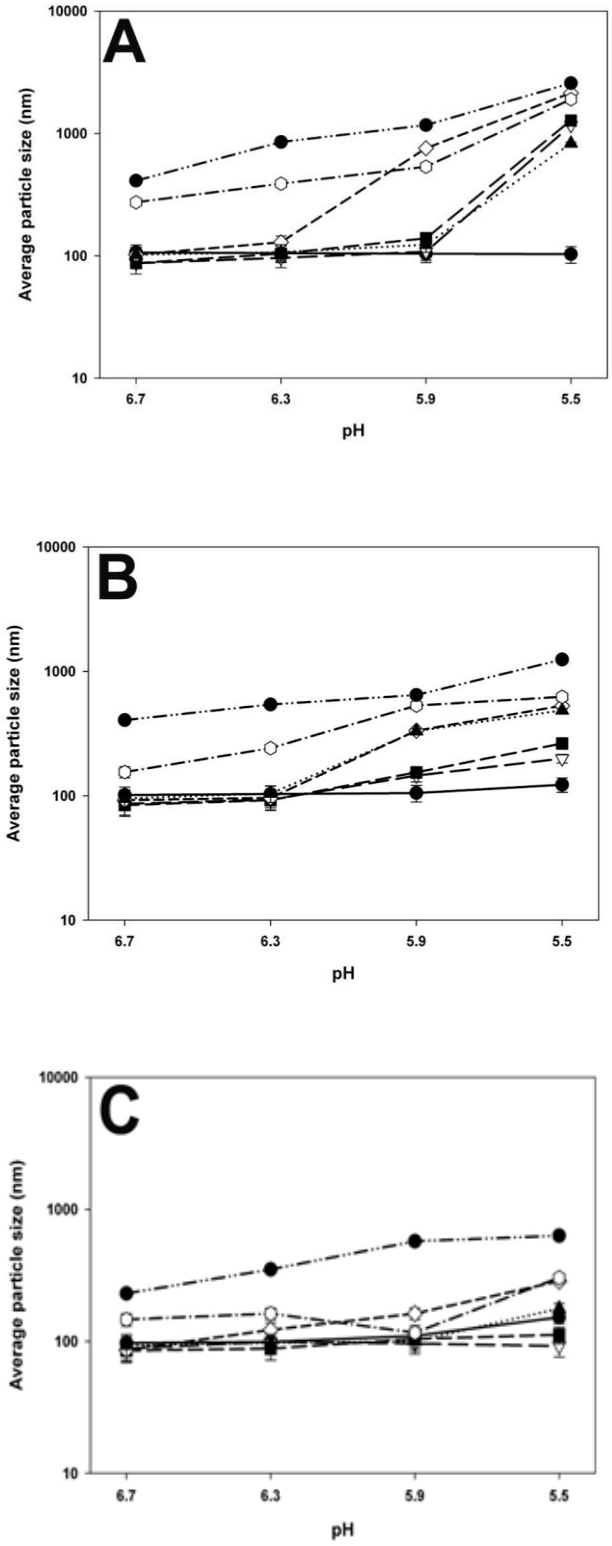


Figure 4.2. Average casein micelle size (nm) in raw skim milk samples chelated with addition of EDTA and citrate at 10, 20 or 30 mM and adjusted to pH of 6.7, 6.3, 5.9 or 5.5 at (A) 15; (B) 30 or (C) 50 °C. Legend: —●— control (raw skim milk); —▽— E10; —■— E20; —◇— E30; ···▲··· C10; —⬡— C20; —●— C30.

Temperature has been reported to have a significant effect on the casein micelle size of casein micelles of raw skim milk when changed from room temperature to pasteurisation temperature (72 °C) and UHT temperature (135-150 °C) (Hougaard, et al., 2009). This is mainly attributed to the attachment of denatured whey proteins onto the casein micelles. The effect of temperature also varies as a function of milk pH. Below pH 2.0, β−Lg exists as a monomer; when closer to its isoelectric point (pH = 5.1), it exists as an octamer at room temperature. Between pH 5.5 and 6.5, β−Lg exists as a dimer at room temperature. Dissociation to a monomer occurs at temperature above 50 °C. Further heating to 65 °C results in unfolding of the structure and subsequent exposure of the free SH group, which is followed by aggregation. The addition of alkali also results in the dissociation of κ−casein which increased with temperature (> pH 6.5) (Anema & Klostermeyer, 1997). However, within the temperature ranges tested (15 to 50 °C), casein micelle size decreased slightly ($P > 0.05$) with the increase of temperature for the samples adjusted to pH 6.7, 6.3 and 5.9, and the casein micelle size only increased with temperature for the sample adjusted to pH 5.5.

At 15 °C, the casein micelle size remained unchanged when pH ≥ 5.9 and then slightly decreased at pH 5.5 with an obvious increase ($P < 0.05$) at pH 5.5 and 50 °C. This was because the casein micelles formed at low pH have more compact structure due to low charge on the proteins (Liu & Guo, 2008).

4.3.3 EFFECT OF TEMPERATURE, pH AND CHELATORS ON THE ZETA POTENTIAL

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles, and is one of the fundamental parameters known to affect stability. Its measurement brings detailed insight into the causes of dispersion, aggregation or flocculation, and can be applied to improve the formulation of dispersions, emulsions and suspensions.

Some information on the surface charges of the micelles may be gained by studying their zeta potentials. The stability of the casein micelle has been attributed in part to the net negative charge residing at the surface (Payens, 1982). An estimation of this surface charge can be derived from knowledge of

the zeta potential of the particle (Darling & Dickson, 1979a, 1979b). As charges on casein micelles are negative, the zeta potentials are also negative. The terms 'increase' and 'decrease', when referring to zeta potential, refer to the absolute magnitude of the zeta potential and are not used algebraically. Measurements of zeta potentials have been made in the past by Dalgleish (1984). Casein micelles in milk at pH 6.7 are generally assumed to remain in suspension due to the hairy layer of κ−casein providing steric and electrostatic repulsion (Wong, et al., 1996).

In this study, average zeta potential of the raw skim milk and that added with chelators (EDTA or citrate, 10, 20 or 30 mM) and then adjusted to pH of 6.3, 5.9 or 5.5 at temperature of 15, 30 or 50 °C is shown in Figure 4.3. The zeta potential was almost unchanged (around −20.7 to −20.0) when temperature increased from 15 to 50 °C for the control sample (raw skim milk at pH 6.7), whereas the zeta−potential increased with the decrease in pH. For example, the zeta potential increased from −20.7 to −23.9 ($P < 0.05$) when pH decreased from 6.7 to 5.5 at 15 °C; it was also increased from −20.3 to −22.6 ($P < 0.05$) and from −20.0 to −21.8 ($P < 0.05$) when pH decreased from 6.7 to 5.5 at temperature of 30 and 50 °C, respectively. This is in agreement with the observations of Darling & Dickson (1979), who reported that zeta potential of casein micelles became less negative with the increase in temperature (from 10 to 50 °C) and pH (from pH 5.3 to 6.9).

With the addition of EDTA or citrate, the change in zeta potential of the raw skim milk is temperature and pH dependant (Figure 4.3). At 15 °C, the zeta potential of the all samples decreased ($P < 0.05$) at all the pH ranges tested, apart from that at pH 6.7, where the zeta potential of a few samples increased ($P > 0.05$) or remain unchanged (Figure 4.3A). At 30 °C, the zeta potential of most samples decreased ($P < 0.05$) apart from those at pH 6.7 and 6.3 that remain unchanged (Figure 4.3B). At 50 °C, the zeta potential of most samples decreased ($P < 0.05$) apart from the samples added with 20 and 30 mM citrate, with which the zeta potential either increased ($P > 0.05$) or remain unchanged at pH of 6.3 and 5.9. The most remarkable reduction in the zeta potential occurred at 15 °C and pH 5.5.

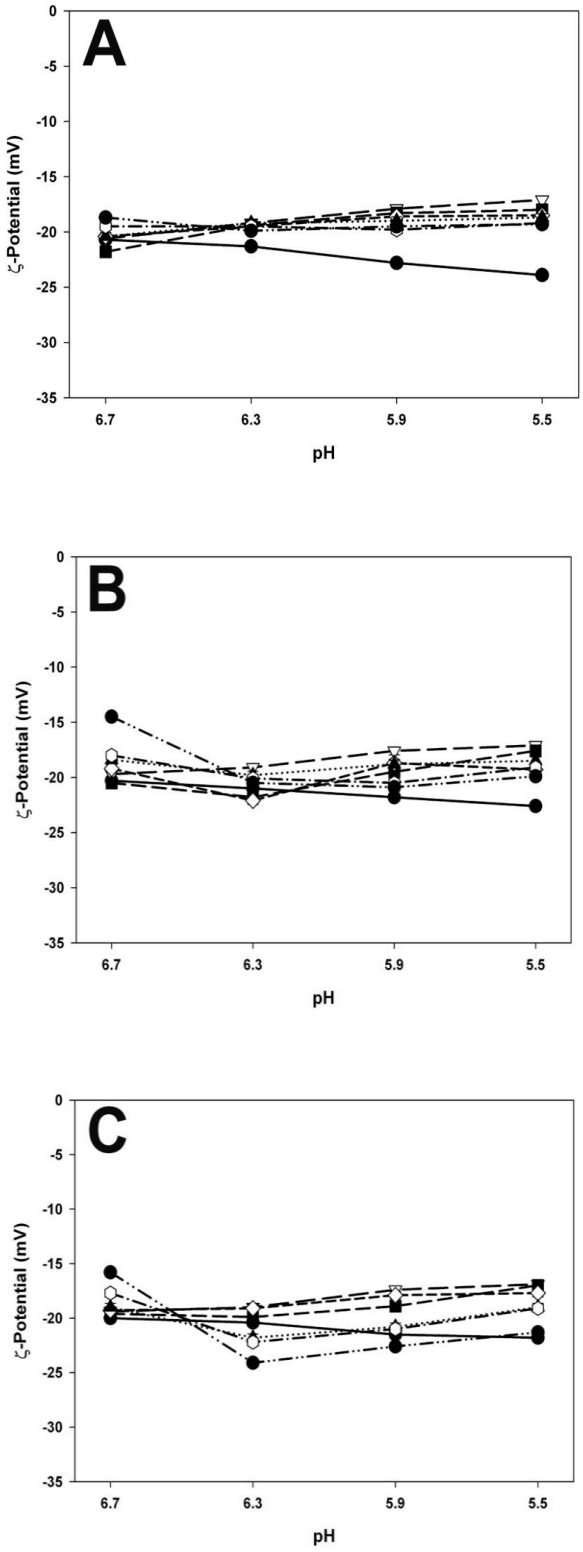


Figure 4.3. Zeta potential (mV) of raw skim milk samples chelated with addition of EDTA and citrate at 10, 20 or 30 mM and adjusted to pH of 6.7, 6.3, 5.9 or 5.5 at (A) 15; (B) 30 or (C) 50 °C. Legend: —●— control (raw skim milk); —▽— E10; -■- E20; —◇— E30; ···▲··· C10; -○-· C20; —●— C30.

With addition of EDTA or citrate at 15°C (Figure 4.3A), the zeta potential decreased ($P > 0.05$) with increasing citrate or EDTA at pH6.7 (control). There is a cross point of zeta potential at pH 6.3; at this point, the zeta potential value was about the same for all EDTA and citrate samples except the control. After this pH, the zeta potential showed similar trend of decrease (less negative) with decrease in pH. At 30°C (Figure 4.3B), the lowest zeta potential was observed for the sample with 30 mM citrate. The zeta potential decreased ($P > 0.05$) for all EDTA and citrate added samples at pH 5.9 and 5.5. At 50 °C (Figure 4.3C), the zeta potential was about same for all samples with EDTA but decreased ($P > 0.05$) for citrate added samples when citrate concentration increased from 10 to 30 mM at pH 6.7, and cross point of pH shifted from 6.3 to about pH 6.5. The zeta potential started to decrease when pH was above 6.3 for all citrate added samples but the amount of decrease was different. At low pH (5.5), the zeta potential of EDTA group was lower ($P > 0.05$) than that of citrate group in general.

4.3.4 EFFECT OF TEMPERATURE, pH AND CHELATORS ON THE Ca DISTRIBUTION

4.3.4.1 Soluble Ca

The soluble Ca content of raw skim milk treated with chelators (EDTA or citrate) and adjusted to pH 6.7, 6.3, 5.9 or 5.5 at temperatures of 15, 30 or 50 °C is shown in Table 4.1.

The soluble Ca decreased ($P < 0.05$) from 13.0 to 12.2 and to 11.4, when the temperature of the raw skim milk increased from 15 to 30 and to 50 °C at pH 6.7 (Control). With pH adjustment, the soluble Ca before addition of chelators was significantly increased ($P < 0.05$) with the decrease of pH; the amount of increase of the soluble Ca was related to the temperature.

After addition of EDTA or citrate into raw skim milk, the soluble Ca decreased with the increase of EDTA from 10 to 30 mM at all pH (6.7, 6.3, 5.9 or 5.5) and temperature ranges (15, 30 or 50 °C). Such a trend only stands for the addition of citrate (10, 20 or 30 mM) at 15 °C. No particular trend was observed for the effect of the added citrate (10, 20 or 30 mM) on the soluble Ca at 30 or 50 °C.

Table 4.1. The soluble Ca from raw skim milk treated with chelators (EDTA or citrate) and adjusted to pH 6.7, 6.3, 5.9 or 5.5 at 15, 30 or 50 °C.

Temperature C°	pH	Soluble Ca [mM]						
			EDTA [mM]			Citrate [mM]		
		Control	10	20	30	10	20	30
15	6.7	13.0	12.6	8.1	7.9	12.6	11.1	10.5
	6.3	13.8	12.7	11.2	10.0	13.4	10.4	7.9
	5.9	14.8	23.7	23.2	18.7	13.9	12.4	10.3
	5.5	17.7	26.4	26.2	24.5	20.0	16.9	13.9
30	6.7	12.2	12.2	11.5	7.8	19.2	5.16	14.7
	6.3	12.7	12.7	10.7	10.2	19.3	23.6	17.5
	5.9	14.5	20.0	17.3	12.0	19.2	8.6	14.7
	5.5	17.5	21.2	23.9	23.3	18.7	16.7	12.3
50	6.7	11.4	11.5	9.4	8.0	13.6	5.1	9.2
	6.3	11.7	11.8	10.2	9.5	15.3	12.3	20.2
	5.9	12.6	23.1	21.0	15.6	21.9	18.9	19.2
	5.5	16.5	25.5	22.9	17.4	18.3	13.8	12.7
SEM			0.934					

Note: The total Ca and soluble Ca of the raw skim milk (~21 °C) is 31.0 ± 0.2 and 12.5 ± 0.2 mM.

Calcium in the milk plays an important role in maintaining the equilibrium between the soluble phase and micellar structure and is influenced by the milk temperature (Davies & White, 1960; Rose, 1968; Pouliot et al., 1989). The results in this study showed that soluble Ca in the milk decreased ($P < 0.05$) when the temperature was increased from 15 to 50 °C. This is due to a shift in Ca from the serum into the micelles as CCP (Davies & White, 1960; Pouliot et al., 1989). The soluble Ca in milk serum decreased very rapidly (< 2 min) when temperature increased from 4 °C to a range of 20 − 90 °C. As the partitioning of those components was influenced by temperature, the Ca phosphate and casein can be exchanged between the micelles and the serum (Davies & White, 1960; Rose, 1968). This exchange demonstrated that casein micelles have a sufficiently open structure to allow movement of casein proteins and the exchange of minerals (Udabage, et al., 2003).

4.3.4.2 Ionic Ca

The effect of pH and chelator at room temperature

The Ionic Ca and pH of raw skim milk before (control) and after addition of chelators (EDTA or citrate) at room temperature (21 ± 0.5 °C), and the ionic Ca in untreated and chelator added milk after adjustment of pH to 6.3, 5.9 or 5.5 is shown in Table 4.2.

Table 4.2. The Ionic Ca before (control) and after addition of chelators (EDTA or citrate, at 10, 20 or 30 mM) at room temperature (21 ± 0.5 °C), after adjustment of pH to 6.3, 5.9 or 5.5.

		Ionic Ca [mM]						
			EDTA [mM]			Citrate [mM]		
		Control	10	20	30	10	20	30
After addition of chelators	Ca^{2+}	1.29	1.06	0.99	0.13	0.10	0.05	0.03
	pH	6.70	6.21	5.60	5.03	6.98	7.18	7.31
After pH adjustment	6.7	1.21	0.66	0.38	0.00	0.27	0.14	0.10
	6.3	2.22	1.12	0.57	0.00	0.63	0.36	0.22
	5.9	5.87	2.45	0.88	0.01	1.98	1.07	0.50
	5.5	14.6	5.77	1.33	0.04	5.80	2.95	1.00

The ionic Ca was reported to be around 1.23 mM (Ran, 2010) to 1.38 mM (Lyster, et al., 1984) at room temperature; this is close to that in this study (1.29 mM). Addition of EDTA (10, 20 or 30 mM) into raw skim milk caused a decrease of pH from 6.7 to 5.0 and a reduction in ionic Ca from 1.29 to 0.13 mM at room temperature (21 ± 0.5 °C); in contrast, addition of citrate (10, 20 or 30 mM) caused an increase of pH from 6.7 to 7.31, and also caused decrease in ionic Ca from 1.29 to 0.03 mM at room temperature (21 ± 0.5 °C).

Adjusting the pH of the samples (with or without addition of chelators) to 6.7, 6.3, 5.9 or 5.5 showed a strong ($P < 0.05$) logarithmic correlation between ionic Ca and the pH (Figure 4.4). It is interesting to notice that the slope of the broken lines (C10, C20 and C30) is greater than that of the solid lines corresponding to the control, E10 and E20. This suggested that pH had a

stronger effect on ionic Ca of the samples that were added with citrate (C10, C20 or C30) than that of the control and that added with EDTA at level of 10 mM (E10) or 20 mM (E20). The exception is for the line of E30 (the dotted line means the ionic Ca is below the detection limit of the meter). This indicated that a different mechanism governs this sample (E30) as shown in Figure 4.1, that a white colour re−appeared after the casein micelles were fully disrupted by the addition of 20 mM of EDTA.

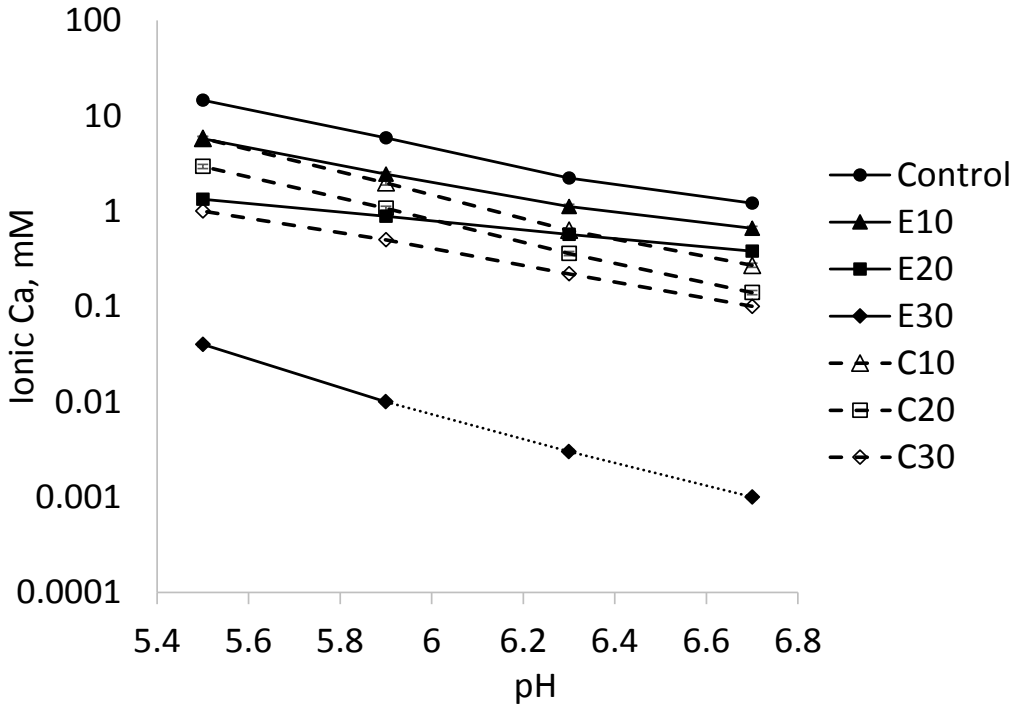


Figure 4.4. Ionic Ca at room temperature (21 ± 0.5 °C) as a function of pH of the raw skim milk (control) and that added with EDTA (E10, E20 and E30), or citrate (C10, C20 and C30).

Addition of EDTA or citrate all caused a decrease in ionic Ca in the milk. The amount of ionic Ca is strongly related to the pH of the milk and the amount of EDTA and citrate added. The overall trend is that the more EDTA or citrate that was added, the lower of the ionic Ca in the milk, and the lower the pH of the milk, the higher the ionic Ca is in the milk. Comparing the ionic Ca of the milk which was added with EDTA and citrate and pH adjusted to 6.7 to 5.5, it was interesting to observe that there is a boundary line in Table 4.2. In the left hand

side of the boundary line, the ionic Ca content of the milk that was added with EDTA (the data in red) was always higher than that was added with citrate (the data in blue); while in the right hand side of the boundary, the ionic Ca content of the milk that was added with EDTA (the data in blue) was always lower than that was added with citrate (the data in red). This suggests that for the combination of pH and chelator that lies in the left hand side of the boundary, the citrate had higher Ca chelating power, while that lies in the right hand side of the boundary, the EDTA had higher Ca chelating power.

The effect of pH and chelators at various temperature

The ionic Ca in raw skim milk with added Ca chelators after pH adjustment at temperature of 15, 30 and 50 °C is shown in the Table 4.3. The ionic Ca decreased ($P < 0.05$) from 1.57 to 1.10 and 0.41 mM, when the temperature of the raw skim milk was increased from 15 to 30 or 50 °C, respectively. The ionic Ca of other samples (that added with chelators and pH adjusted) followed one obvious trend with temperature that the ionic Ca decreases with the increase in temperature. The extent of change in ionic Ca with pH was non-linear and temperature dependant. At 15 °C, the ionic Ca content increased ($P < 0.05$) from 1.57 mM to 2.15, 6.09 and 13.3 mM when pH decreased from 6.7 to 6.3, 5.9 and 5.5, respectively. At 30 °C, the ionic Ca increased ($P < 0.05$) slightly from 1.10 to 1.83 and 3.73 mM as the pH was adjusted from 6.7 to 6.3 and 5.9, and then increased sharply ($P < 0.05$) to 12.2 mM when the pH was adjusted to 5.5. At 50 °C, the ionic Ca followed the same trend as that at 30 °C and increased ($P < 0.05$) from 0.41 (control) to 1.11, 1.90 and 4.01 mM when pH was adjusted from 6.7 to 6.3, 5.9 and 5.5.

The addition of EDTA or citrate reduced ionic Ca in the milk for all the temperatures. The more chelator concentration was used, the lower was the ionic Ca. The “boundary” observed in the room temperature also existed in all the temperatures tested (15, 30 and 50 °C). The boundary line shifted upwards with the increase in temperature. This indicated that, comparing the chelating power of EDTA and citrate at different temperatures, increasing temperature favours EDTA.

The ionic Ca content at 15 °C and pH 6.7 was 1.57 mM which was in the same range of 1.38 −1.58 mM as reported by Gao (2010). When samples were pH adjusted to the range of 6.7 to 5.5 after addition of EDTA or citrate, the ionic Ca in both groups decreased ($P < 0.05$). The more EDTA or citrate that was added, the more decrease in ionic Ca. This result was in agreement with Augustin and Clarke (1990) and Singh, et al. (1995), who reported that Ca chelators such as citrate or EDTA could shift protein−mineral equilibrium by the affinity for Ca ions and interaction with the amino acids of caseins, leading to a decrease in the concentration of free Ca ions in the milk.

Table 4.3. Ionic Ca of control (native raw skim milk) and raw skim milk samples that were added with chelators (EDTA or citrate, at levels of 10, 20 or 30 mM) and pH adjusted to 6.7, 6.3, 5.9 or 5.5 at temperature of 15, 30 or 50 °C.

Temperature C°	pH	Ionic Ca [mM]						
			EDTA [mM]			Citrate [mM]		
		Control	10	20	30	10	20	30
15	6.7	1.57	0.80	0.43	0.00	0.28	0.16	0.11
	6.3	2.15	1.10	0.50	0.01	0.59	0.35	0.22
	5.9	6.09	2.46	0.84	0.01	1.96	1.04	0.48
	5.5	13.3	5.53	1.21	0.03	4.90	2.53	0.88
30	6.7	1.10	0.52	0.33	0.01	0.23	0.14	0.10
	6.3	1.83	0.98	0.60	0.01	0.61	0.38	0.24
	5.9	3.73	2.15	0.85	0.02	1.77	0.99	0.52
	5.5	12.2	4.83	1.29	0.05	5.60	2.98	1.08
50	6.7	0.41	0.19	0.11	0.01	0.14	0.11	0.09
	6.3	1.11	0.48	0.38	0.02	0.42	0.23	0.16
	5.9	1.90	1.02	0.57	0.03	0.83	0.61	0.37
	5.5	4.01	2.01	0.81	0.06	2.17	1.42	0.64
SEM					0.151			

The effect of EDTA on the mineral equilibrium was investigated by Keowmaneechai and McClements (2002), who reported that EDTA had strong

Ca binding capacity. The impact of different Ca chelator on the mineral equilibria and casein micelle structure might be different, as affinity for Ca ions (i.e. association and solubility constants) and interaction with the amino acids of caseins might be different (Holt, 1981; 2004; Mekmene, et al., 2009). This was observed in this study that the citrate presented more of an effect on the decrease of Ca ions than EDTA at low concentration (≤ 20 mM) and pH close to native (i.e. pH 6.7 and 6.3), but when 30 mM of EDTA was added, the colour of the milk was changed back to white as can be observed in Figure 4.1. This could be linked to the report that Ca chelators may also have limitation of Ca binding ability at a certain concentration, as they can chelate CCP from the casein micelle to a level at which the integrity of the micelle structure is lost (Griffin, et al., 1988). From this study, it was indicated that 20 mM of EDTA or 30 mM of citrate was required for fully chelate CCP in the casein micelle in the raw skim milk at room temperature (21 ± 0.5 °C).

The increase of ionic Ca content when the pH of the raw skim milk was reduced could be induced by the surface hydrophobicity of micelles (Jean, et al., 2006; Law, 1996) and a progressive solubilisation of CCP and other colloidal salts from the micelles which took place at low pH. The effect of pH on the milk was also reported by Gaucheron (2005), when milk was adjusted to low pH, there were few reactions involved. Firstly, the proton addition or in situ production; secondly, it was the association of H^+ with citrate Pi and Po that induced the solubilisation of micellar Ca phosphate, resulted in disturbed salt balance, increase of ionic Ca concentration and increase in the ionic strength, leading to the hydration and their zeta potential decrease.

Increase in milk temperature caused a decrease in ionic Ca. This was because the β−Lg as one of majority portion, which represents around half of the whey proteins (Thompson, et al., 2009) and interacts with other milk proteins during thermal processing, formed protein aggregates. The irreversible aggregation with itself and other molecules such as κ−casein and α−La occurred at elevated temperatures (Visser & Jeurnink, 1997), but the structure and aggregation state of β−Lg is also strongly dependent on pH (Creamer & MacGibbon, 1996; Sawyer, et al., 2002).

In general, it is known the casein micelles could dissociate to various degrees when the pH of the milk was decreased (Matia−Merino & Singh, 2007). The Ca phosphate begins to dissociate when the pH is decreased from normal milk (pH 6.7) (Van Hooydonk, et al., 1986). Its dissociation can cause dissociation of individual casein molecules (Holt, et al., 1986) and dissociation of casein micelles or CCP including the Ca movement from Ca ions, Ca−linked to casein, and Ca with phosphate in CCP, leading to the change of casein micelle size. So the pH range is critical for the micelle change. The report by Rabiller−Baudry, et al. (2009) showed that pH in between 5.5−6.7 has obviously affected the Ca^{2+} and Ca complex with caseins.

Addition of Ca chelators affected casein micelle size, zeta potential, soluble Ca and ionic Ca. Addition of EDTA and citrate caused an increase in casein micelle size, and a reduction in soluble and ionic Ca. When Ca chelator (e.g. EDTA) is added to the milk system, EDTA reacts with Ca ions to form very stable complexes−Ca EDTA, which has strong capacity to bind Ca ions. The chelator therefore shifts the casein−mineral equilibrium, dissolute CCP from micelle and releases specific caseins from the micelles (Holt, 1992; Gaucheron, 2005; Panouille, et al., 2004). Casein micelles may eventually dissociate into smaller clusters upon addition of chelators (Panouille, et al., 2004; Marchin, et al., 2007; Griffin, et al., 1988; Udabage, et al., 2000).

4.4 Conclusions

Temperature and pH of the raw skim milk and the addition of chelating agents had a significant effect on soluble and ionic Ca in the raw skim milk, which affected casein micelle size and zeta potential in different ways. An increase in temperature from 15 °C to 50 °C led to a decrease in soluble and ionic Ca, and a decrease in zeta potential but the casein micelle size remained unchanged. Reducing the pH of the raw skim milk from 6.7 to 5.5, resulted in an increase in zeta potential, and an increase in soluble and ionic Ca. Casein micelle size was also pH dependant. At pH 6.7 and 6.3, casein micelle size was not affected by the temperature, while at low pH, in particular at pH 5.5, the casein micelle size increased with the increase of temperature. Addition of EDTA or citrate caused

a decrease in ionic Ca in the raw skim milk and resulted in complete disruption of casein micelles when 20 mM of EDTA or 30 mM of citrate was added.

Overall, casein micelle size, zeta potential, soluble and ionic Ca in the raw skim milk is affected by and can be manipulated to certain extent by the temperature, pH and the addition of chelating agents.

Chapter 5. **Lower UF Temperature Improves Membrane Performance and Emulsifying Properties of MPC**[1]

5.1 Introduction

MPC, produced from skim milk by membrane concentration and drying, and contains all the caseins and whey proteins present in milk with low concentrations of lactose and minerals. Such a MPC has been used as a functional ingredient in many food applications. For commercial and technological reasons, MPC is normally converted into powders (Faka, et al., 2009) and has been used for a number of reasons in various dairy products. In recent years, a number of milk powders with high protein content (50 − 80 %) has been developed using membrane technologies (Premaratne & Cousin 1991). UF or a combination of UF with diafiltration (DF) is widely used to separate and concentrate milk proteins (ST−Gelais, et al., 1992). To be useful as functional ingredients, MPC powders should exhibit good functionality such as emulsification, high solubility and heat stability in food products. Conditions during production of MPC powder apparently affect their composition, most importantly mineral composition and protein concentration and structure, which in turn may influence their physical functionality (Sikand, et al., 2011). A recent work by Ye (2011) has, for example, demonstrated that emulsifying properties of MPC were influenced by casein micelle size. The size of casein micelles was obviously affected by reduction of Ca content in the MPCs−the lower the Ca content in MPC the smaller the particle (droplets) size in emulsion leading to a better functionality.

Various processing conditions during concentration of milk proteins are known

[1] X. Luo, L. Ramchandran, T. Vasiljevic. (2015). Lower Ultrafiltration Temperature Improves Membrane Performance and Emulsifying Properties of Milk Protein Concentrates. Journal of Dairy Science and Technology, 95 (1), 15-31.

to affect their physical functionalities. For example, temperature of operation is an important parameter for skim milk concentration by a UF process. When milk proteins are subjected to thermal processing, depending on the heating conditions, whey proteins may undergo subtle structural changes or even reversible denaturation (Raikos, 2010). Denatured whey proteins can interact with themselves and /or with κ−casein to form heat−induced protein aggregates (Donato, et al., 2007). Kinetics of protein denaturation and aggregation is affected by heating conditions and the environment, such as pH and ionic strength, which in turn affects the rate and extent of protein denaturation and extent of subsequent interactions of the whey proteins with the casein micelles, resulting in the change of a micelle size (Anema & Li, 2003b; Anema et al., 2004). UF at high temperatures above 70 °C (Lalande & Tissier, 1985) favours membrane filtration by lowering liquid viscosity (Pace, et al., 1976; ST−Gelais, et al., 1992) and increasing mass transport across the membrane (Marcelo & Rizvi 2008). However, a high temperature could accelerate membrane fouling caused by protein denaturation leading to protein interactions and gelling on membrane surfaces (Gautam, 1994).

The effect of heat on protein denaturation and aggregation has been studied extensively and has been well reviewed (Considine, et al., 2007; Raikos, 2010). However, the investigation of functional properties as affected by casein micelle size and heat effect mainly focused on using reconstituted milk powders (Anema & Li, 2003a). The change of the casein micelle size induced by different heating conditions was reported by using centrifuged skim milk (Ono, et al., 1999). The production of MPC from UF retentate obtained by processing skim milk at 10 and 50 °C has been reported by Havea (2006) and Syrios, et al. (2011) but the effect on the properties of MPC has not been studied. Consequently, comprehensive information on the effect of temperature of UF processing on MPC functional performance is scarce.

This work investigated the effect of the processing temperature during a five-fold concentration of skim milk by UF on the functionality of the MPC and membrane performance. The results from this study may help establish a foundation for optimizing a process regime for the production of MPC with improved physical functionality.

5.2 Materials and Methods

5.2.1 PREPARATION OF MILK CONCENTRATES

The milk used for this study was skim milk (0.3% fat) commercially pasteurized (72 °C / 15 s) provided by a local supplier (Woolworths, Melbourne, Australia).

The skim milk was heated and maintained at 15, 30 or 50 ˚C, respectively, using a water tank. A membrane module (SEPA CF) and polyethersulfone (PES) membrane (190 x 140 mm) with a molecular cut−off of 20 KDa were both purchased from SterliTech Corporation (Kent, WA, USA). The set up was conditioned to the temperature of operation by running Milli−Q water at the same processing temperatures before running the milk sample. The skim milk was pumped through the membrane module at a pressure of 2 bar using a hydra−cell pump (T−VERTER 220V 1.5 KW 2HP, Wanner Engineering Inc., Minneapolis, MN, USA) and the concentrate was recycled into the feed container until the volume was reduced to 1 / 5 of its original volume (volume concentration ratio = 5). This was monitored by measuring the volumes of collected permeate during UF. The pH of all retentates was assessed at the end of the process and was in the range 6.4 − 6.5.

Samples of permeate and retentate, collected at the start (0 h) and at the end of operation (5-8 h, depends on the temperature), were analysed immediately for casein micelle size, zeta potential and conformational changes of proteins. A part of the concentrated samples was stored at −20 °C freezer and used for Ca and protein analyses, and the remaining part of the frozen concentrates was freeze dried (Dynavac, Model FD 300, Dynavac Eng. Pty. Ltd., Melbourne, Australia) and reconstituted for the functionality (e.g. emulsion) analysis.

New membrane sheets were used for each run, which were wetted with Milli−Q water over night before use. The samples were frozen at −20 °C before freeze drying.

5.3 Results and Discussion

5.3.1 CHANGES IN MICELLE SIZE AND ZETA POTENTIAL

The casein micelle size distribution of skim milk and skim milk concentrates obtained by UF at different temperatures is shown in Figure 5.1.

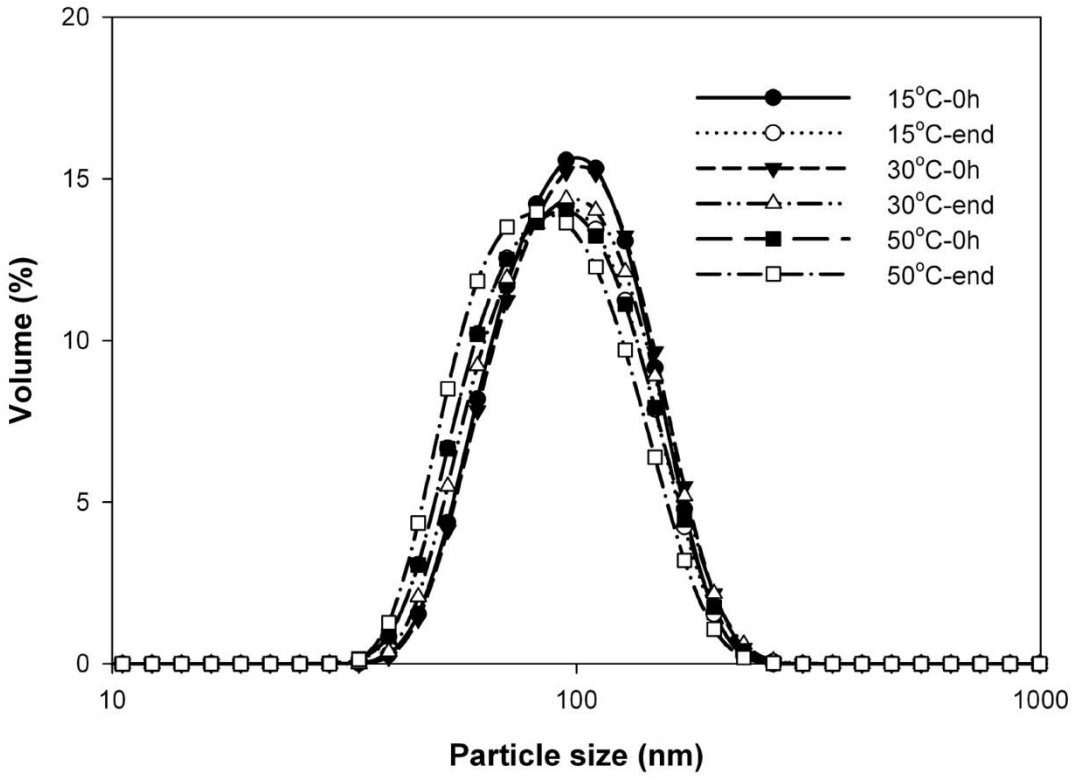


Figure 5.1. The casein micelle size distribution of retentates obtained by membrane UF operated at 15, 30 or 50 °C.

Initially the average casein micelle size was not affected ($P \geq 0.05$) by processing temperature and ranged from 87.4 ± 1.8 nm to 92.7 ± 3.8 nm for the milk equilibrated at 15 and 50 °C, respectively. Furthermore, the average casein micelle size did not change noticeably ($P \geq 0.05$) after concentration at 15 or 30 °C. This is in agreement with the results of Martin, et al. (2010), who also reported no major change in micelle size during UF / DF at 10 ˚C. On the contrary, a significant decrease ($P < 0.05$) in casein micelle size (from 92.7 to 83.6 nm) was observed (Figure 5.1) when UF was carried out at 50 ˚C. This

contradicts the observation by Green, et al. (1984), who reported that the size of casein micelles did not change during concentration of skim milk by UF at 50 °C. The observed casein micelle size decrease at 50 ˚C may be contributed to plasmin related dissociation of caseins. Plasmin is an endogenous protease present in milk and is relatively heat stable thus is not fully inactivated during commercial pasteurisation. Furthermore its activity optimum is close to physiological temperatures and increases in heat treated milk (Kelly & McSweeney, 2003). Since commercially pasteurized skim milk was used in this experimentation, it is possible that plasmin present in the sample remained active and continued hydrolysing caseins at elevated temperatures during ultrafiltration.

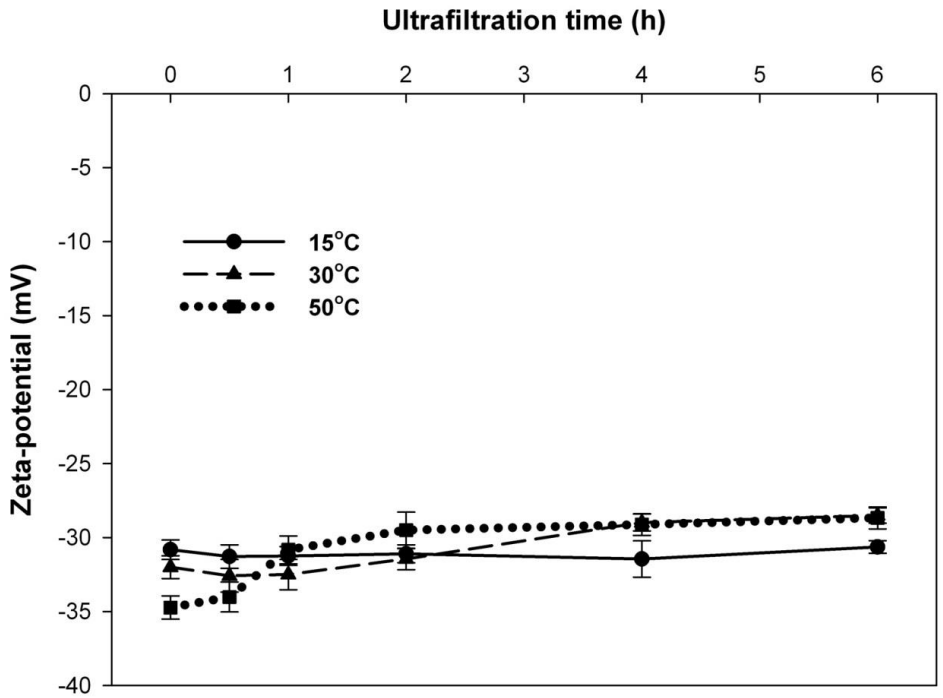


Figure 5.2. Zeta potential as a function of concentration time of concentrated skim milk obtained by UF at 15, 30 or 50 °C, respectively.

Zeta potential results (Figure 5.2) showed that the proteins were negatively charged with no substantial change ($P > 0.05$) during concentration at 15 °C. However, at 30 and 50 °C, a decrease in the net negative charge in the retentates in comparison to those in their respective feeds was observed. This

result is in agreement with the observations of Darling and Dickson (1979a, 1979b) that zeta potential of casein micelles increased with increase in temperature from 10 to 50 °C. This reduction in net negative charge on casein micelles could also be contributed by possible modifications of surface charge as a consequence of hydrolysis by plasmin activity at temperatures closer to its activity optimum (Crudden, et al., 2005).

5.3.2 CHANGES IN Ca CONTENT

The Ca content of retentate obtained during UF of skim milk operated at 15, 30 or 50 °C is shown in Table 5.1. The Ca content of the retentate increased with elevation of the operation temperature (from 15 to 50 °C) with corresponding decrease in the permeate. On an average, UF removed 24.9 % of the Ca from skim milk into permeate at 15 °C compared to 20.2 % at 30 °C and 16.6 % at 50 °C, indicating that higher UF temperature coincides with less efficient Ca removal. Syrios, et al. (2011) also indicated that increased UF temperature resulted in reduced ionic Ca in the permeate when heating the skim milk to 50 °C prior to UF. Similar observations have been made by Chandrapala, et al. (2010 a, b), Lewis (2011), On−Nom, et al. (2010), Premaratne and Cousin (1991), Rose and Tessier (1959) and Vasiljevic and Jelen (1999).

Table 5.1. Total protein (g / 100 g) and Ca (mM) contents of retentates and permeates obtained during 5 times concentration of skim milk by UF at 15, 30 or 50 °C.

UF temperature °C	Retentate		Permeate	
	Total Ca	Total protein	Total Ca	Total protein
15	99.7 ± 1.4^{a}	15.9 ± 0.04^{b}	6.90 ± 0.99^{c}	0.18 ± 0.01^{a}
30	110.3 ± 2.1^{b}	15.4 ± 0.02^{a}	5.60 ± 0.54^{b}	0.22 ± 0.01^{b}
50	111.7 ± 1.5^{b}	16.4 ± 0.1^{c}	4.60 ± 0.32^{a}	0.26 ± 0.01^{b}

Note: All values presented are the mean ± standard deviation (n = 6). Means in the same column with different letters are significantly different (P < 0.05). Total protein of skim milk feed was 3.3 ± 0.11 g / 100 ml. Total Ca content of skim milk feed was 26.79 ± 1.17 mM.

During UF, about 10 mM soluble Ca (30 % of total Ca), which is in the diffusible form, can pass through the membrane and accumulate in the permeate (Holt, 1981). Removal of Ca during UF would result in some modifications in the salt system of milk such as solubilisation of colloidal Ca phosphate and migration of Ca from the micelles (Syrios, et al., 2011). These changes in the salt system could also have affected the structure of casein micelles in milk (ST−Gelais, et al., 1992). It follows that a relatively larger proportion of Ca was removed through the permeate during UF at 15 °C as compared to 30 and 50 °C (Table 5.1). Further, small quantities of Ca were deposited on the membrane surface during UF (Table 5.2), the amount being similar at 15 and 30 °C but increasing ($P < 0.05$) at 50 °C. It could be concluded that temperature of UF not only influenced the extent of removal of Ca into the permeate, but also contributed to the extent of membrane fouling.

Table 5.2. Protein and Ca content retained on the membrane surface during 5 times concentration of skim milk by UF conducted at 15, 30 or 50°C.

UF temperature °C	Protein $mg.cm^{-2}$	Ca $\mu g.cm^{-2}$
15	1.34 ± 0.001^{a}	2.5 ± 0.01^{a}
30	1.25 ± 0.001^{b}	2.0 ± 0.01^{a}
50	2.18 ± 0.003^{c}	3.0 ± 0.01^{b}

Note: All values presented are the mean± standard deviation (n = 6). Means in the same column with different letters are significantly different ($P < 0.05$).

5.3.3 CHANGES IN MILK PROTEINS

The change in protein content at the end of skim milk UF at different temperatures is shown in Table 5.1. As expected, the protein content of the concentrate increased as a consequence of UF. Further analysis of the protein distribution in permeate as well as on the membrane revealed that a proportion of the total protein was retained on the membrane surface (Table 5.2) while negligible quantities of protein passed into permeates (Table 5.1). These results indicated that proteins were likely the major foulants during UF of skim milk,

being significantly greater ($P < 0.05$) at 50 °C than at 30 or 15 °C.

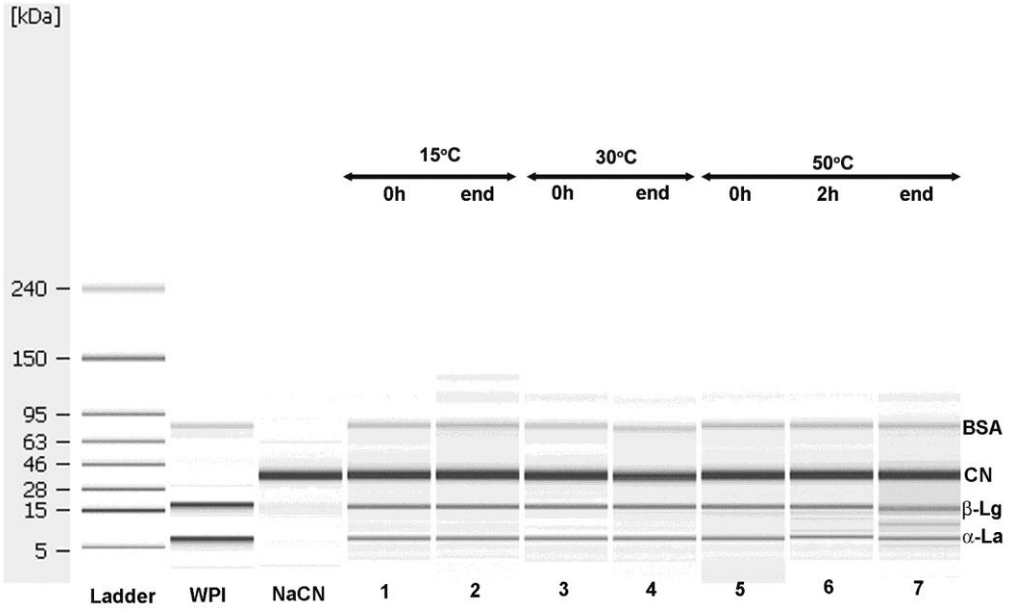


Figure 5.3. Electrophoretic patterns under non-reducing conditions of feed and retentate samples obtained by UF of skim milk operated at 15, 30 or 50 °C, at the beginning (0h – feed), after 2 h and at the end of concentration. The ladder is composed from the external standard included in the protein kit (Agilent). The lanes WPI and sodium caseinate were obtained by using commercial whey protein isolate and sodium caseinate (NaCN), respectively, as external standards. The bands indicated present BSA – bovine serum albumin, CN – caseins, β-Lg – β-lactoglobulin, and α-La – α-lactalbumin.

Electrophoretic patterns obtained (Figure 5.3) showed the protein profiles of the retentates obtained during UF (at time 0, 2h, and at the end of operation) at 15, 30 and 50 °C, respectively. In the pattern obtained by the Bioanalyser, the bands representing α−casein, β−casein, κ−casein, β−Lg and α−La are usually displayed across values from 12 − 46 kDa. Concentration of skim milk by UF resulted in formation of some low molecular weight compounds (lanes 6 and 7), particularly when operated at 50 °C. Dalgleish and Law (1988, 1989) have indicated that small changes in colloidal Ca phosphate and other Ca phosphate nanoclusters can induce rearrangements within casein micelles that could result in solubilisation of some of the casein fractions from within the micelle. Further, the plasmin−assisted dissociation of caseins could also result in release of low molecular weight fractions. Given that plasmin is associated with caseins, increase in protein concentration in the retentate could have resulted

in casein hydrolysis and release of low molecular weight peptides particularly at 50 °C (Dupont, et al., 2013). Plasmin preferentially cleaves β−casein, while κ−casein basically remains intact, thus it still may provide stability to the casein micelle (Kelly & McSweeney, 2003). Activity of plasmin has also been correlated to concentration of β−Lg (Aaltonen & Ollikainen, 2011) which was also concentrated in the retentate. Thus, overall high plasmin activity and plasmin−induced changes in zeta potential (Figure 5.2) might have promoted micellar disintegration (Crudden, et al., 2005), which also supports earlier observation related to reduced casein micelle size in resultant MPC powder obtained by UF at 50 °C.

Table 5.3. Average peak areas at specific wave numbers obtained from peak tables of interferograms for skim milk at the start (0 h) of UF and retentate obtained after 5 times concentration by UF at 15, 30 or 50 °C.

UF operation temperature °C	Sampling time	Wave number cm^{-1}
		1635
15	0 h	12.85
	End	12.23
30	0 h	11.39
	End	11.76
50	0 h	10.86
	End	12.20

Note: All values presented are the means obtained from 6 interferograms.

Tabble 5.3 shows the major peak areas of interest obtained from interferograms of milk and corresponding MPCs obtained at the various temperatures of UF. Changing the temperature of operation from 15 to 50 °C resulted in little change in the backbone structure of milk proteins (observed between 1640 and 1660 cm^{-1}) with β−sheet structures (1635 cm^{-1}) predominating at 30 and 50 °C in all tested samples. Intensity of β−sheets in MPCs decreased when UF was carried out at 15 °C, increasing slightly at 30 °C but then augmenting substantially at 50 °C. Such an enhancement of β−sheet intensity particularly at 50 °C is indicative

of a structural change in proteins likely as a consequence of changes in surface properties such as zeta potential (Figure 5.2) due to plasmin activity that could have resulted in a partial dissociation of the micelles and thereby reduced casein micelle size.

5.3.4 MPC SOLUBILITY AND HEAT STABILITY

Figure 5.4 shows the solubility (%) of freeze−dried MPC powders obtained at 15, 30 and 50 °C. As noted, operation temperature affected solubility of the MPCs slightly, with solubility of all samples below 80 % and declining with increase of processing temperature. Our observation is similar to other reports which poor solubility of MPC powders attributed to their high protein content (Fang, et al., 2011). Depletion of minerals and resulting dissociation of casein micelles are believed to contribute to the poor solubility of MPC powders (Sikand, et al., 2011). However, no such variations in solubility were observed in the current study (Figure 5.4), probably because the MPC powders were freeze dried, while those reported so far used spray dried MPC powders.

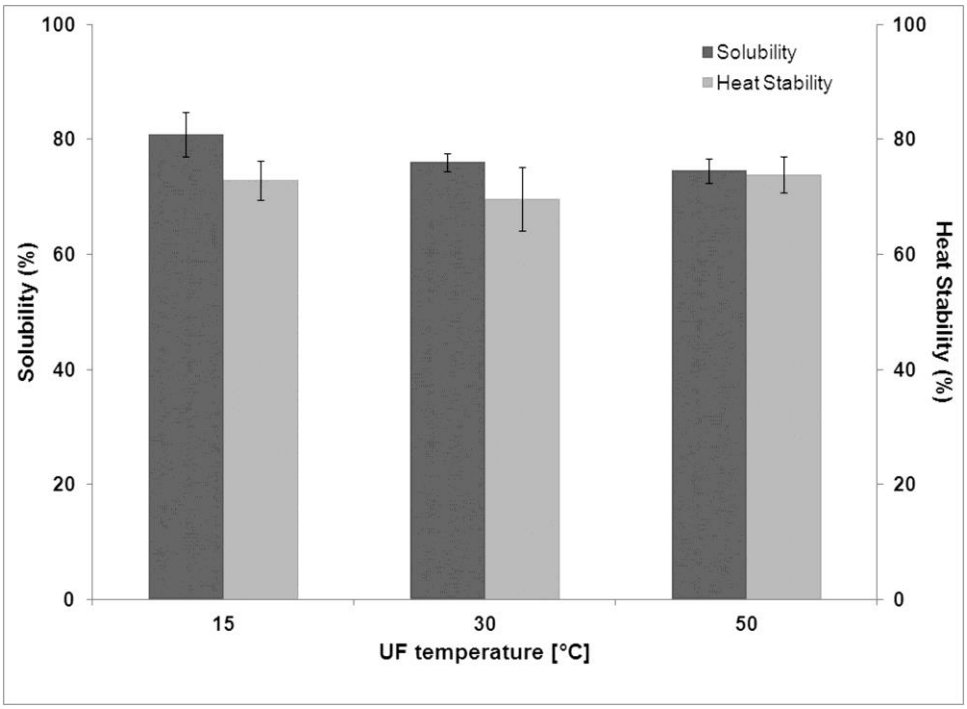


Figure 5.4. Solubility and heat stability of MPCs obtained by UF of skim milk at 15, 30 and 50 °C.

Similar to solubility, only slight differences in heat stability among the powders were noted (P ≥ 0.05, Figure 5.4) across the tested processing temperatures. Although many factors are known to affect the heat stability of milk proteins, such as pH, proteins, salts and processing conditions (O'Connel & Fox, 2003), there are no reports on the heat stability of MPC powders. Considering that there were no major conformational changes to proteins observed (Table 5.3), the similarity in heat stability can thus be expected.

5.3.5 EMULSIFYING PROPERTIES OF MPC

The emulsion activity index (EAI) and emulsion stability index (ESI) (Figure 5.5) indicated that there is no significant difference in EAI and ESI of MPC obtained at 15, 30 and 50 °C.

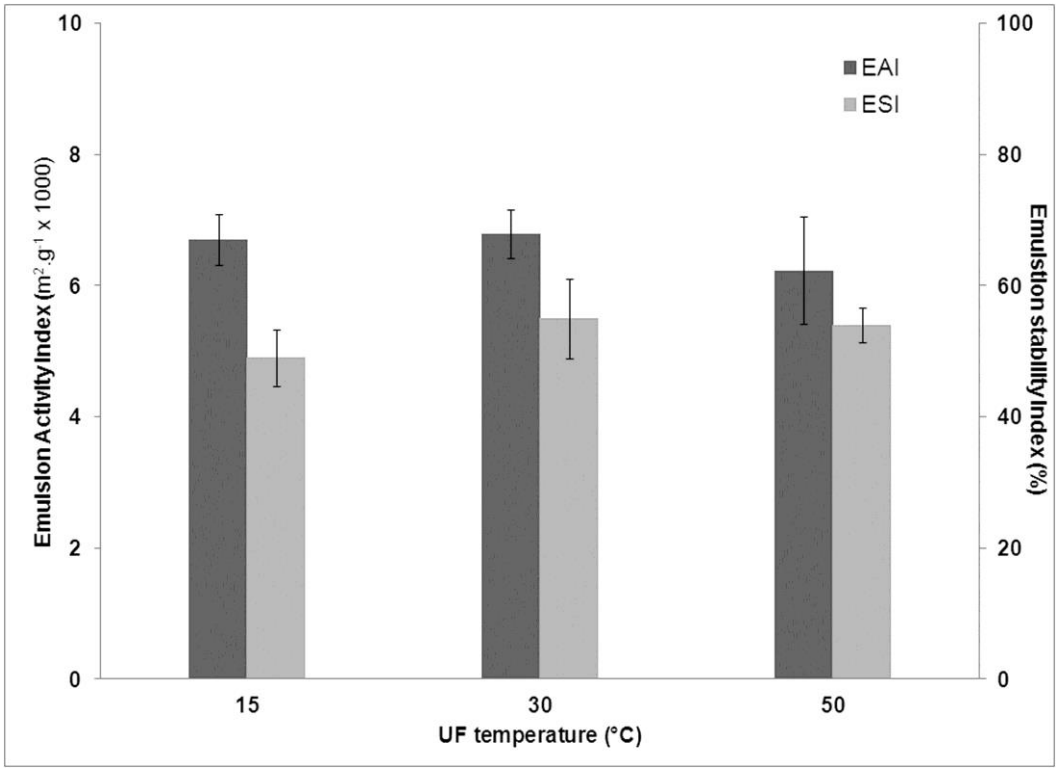


Figure 5.5. The Emulsion Activity Index (**EAI**) and Emulsion Stability Index (**ESI**) of MPCs obtained by UF of skim milk at 15, 30 or 50 °C.

The emulsion properties are known to be influenced by pH, Ca concentration, protein concentration and casein micelle size (Hill, 1996). The casein micelle size in particular is understood to play an important role in the emulsifying capability of proteins (Ye, 2011). The emulsifying properties and emulsion stability have been related to ionic strength and may be improved by a low ionic strength without affecting the emulsion capacity (Mena−Casanova & Totosaus, 2011), as was also observed in our study. The lower the Ca content in MPC, the smaller the casein micelle size in dispersion and droplet size in emulsion, leading to a finer and more stable emulsion. Changes in Ca^{2+} influence the extent of their binding with phosphoserine of caseins leading to an increase or decrease in electrostatic repulsion between the molecules, which in turn influenced the emulsion properties (Ye & Singh, 2001). The results in this study showed that Ca migration during the UF can be affected by operation temperature (Table 5.1). Thus, Ca reduction could be achieved by concentrating proteins during the UF with minimal changes to protein conformation and thus without a major impact on their emulsifying properties.

5.3.6 MEMBRANE PERFORMANCE DURING UF

The membrane performance was monitored by a flux measurement (Figure 5.6) and SEM imaging presented in Figure 5.7. In general the flux declined steadily with the time during continuous UF. The rate of a flux decline was similar at 15 and 30 °C. However, at 50 ˚C the rate of decline was rapid ($P < 0.05$) during the initial 3 h of operation, after which it was steadied. Increased deposition of Ca and protein (Table 5.2) could have contributed to membrane fouling, leading to a decreased flux. It can be seen that UF operating at 15 °C gives best membrane performance.

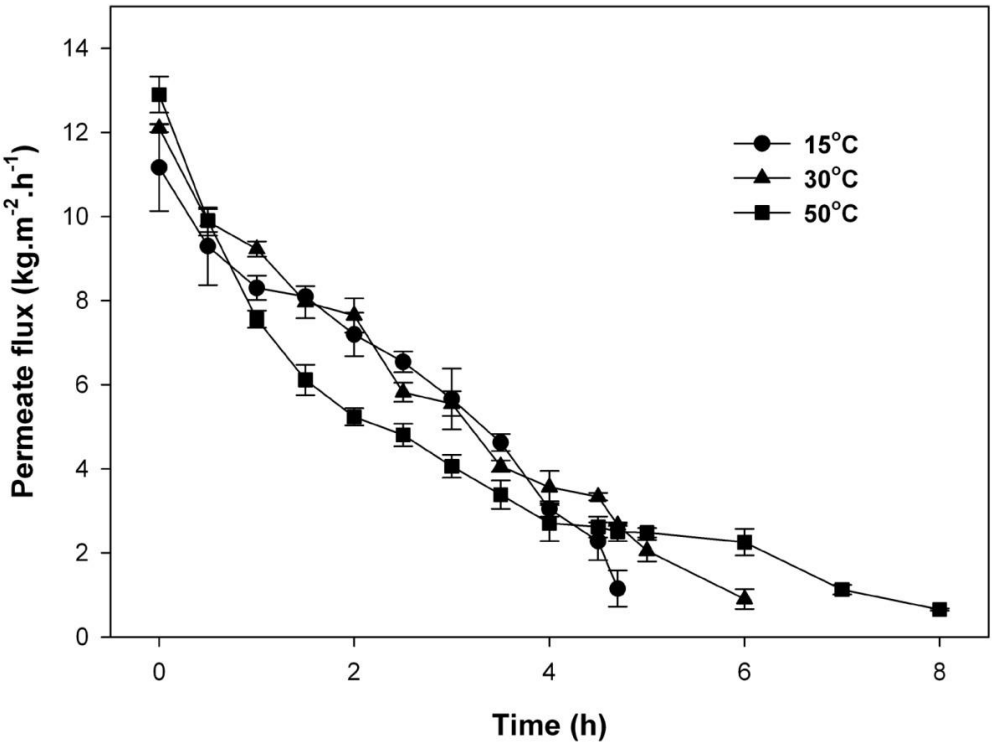


Figure 5.6. The permeate flux during UF of skim milk at 15, 30 and 50 °C.

SEM images (Figure 5.7) indicated that the active surface of membranes operated at 15 ˚C was cleaner as observed by the presence of some clear pores and lack of fouling in the cross sectional images. In contrast, membrane obtained after operation at 50 ˚C had some white particles sticking to the surface which could not be removed by washing, and the pores were blocked and deformed in some areas as shown in the cross section SEM images supporting the earlier finding that greater fouling occurred at higher temperatures. Analysis of the membranes (Table 5.2, Figure 5.7) showed that more protein and Ca deposited on the membrane operated at 50 ˚C than that operated at 15 or 30 ˚C, with proteins being greater contributor than Ca at 50 °C. Thus, both proteins and Ca in milk contributed to the loss of membrane performance as a consequence of fouling, more so at 50 °C than at 15 °C.

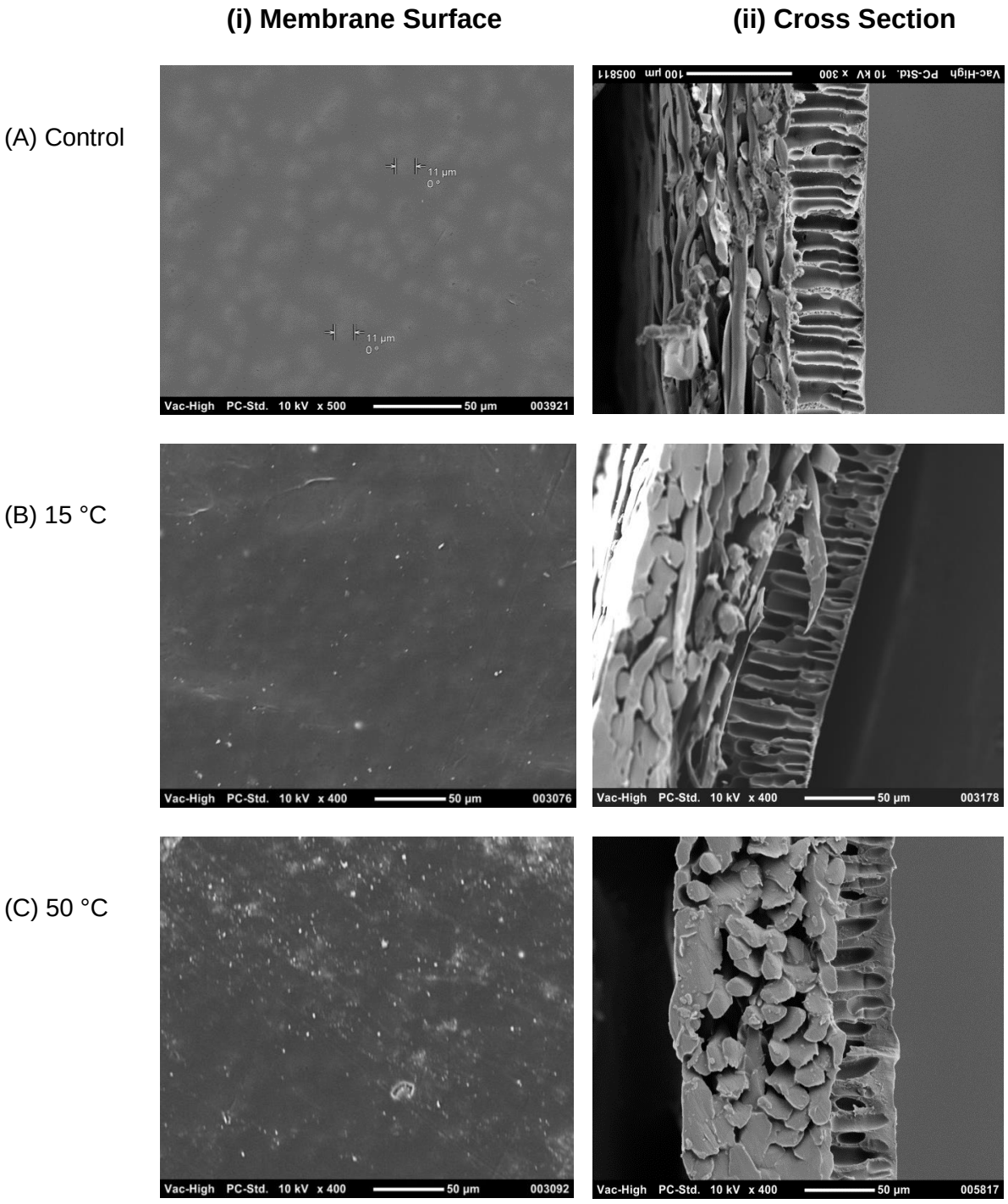


Figure 5.7. The SEM images of membrane surface and cross section of new membrane (A), after skim milk UF at 15 °C (B), and at 50 °C (C).

5.4 Conclusion

Careful selection of operation parameters can assist in producing MPC with a tailored functionality. Operation temperature is one such parameter that was found to have significant effect on the average casein micelle size of the MPC obtained. Overall, lower temperature of operation (15 °C) produced MPC with a comparable small casein micelle size, better emulsion functionality and less membrane fouling without much alteration in protein conformation, solubility and heat stability, in comparison to those produced at higher temperature (50 °C) of UF. This could be due to a greater removal of Ca in permeate and likely with some involvement of plasmin activity. Further investigation is thus warranted to establish how additional manipulations of operating conditions, including feed modifications via pH adjustments, could govern the mineral balance in milk and consequently the functionality of the MPC produced by UF.

Chapter 6. **Effect of Adjusted pH Prior to UF of Skim Milk on Functionality of MPC[2]**

6.1 Introduction

MPC is a product obtained by membrane separation processing, and contains all the caseins and whey proteins present in skim milk. This product provides nutritional value and allowing for texture modifications, enhanced emulsification capacity, which has been used in cheese, processed cheese and yoghurt manufacturing and are commercially available in a range of protein levels (Ye, 2011). Physical functionality of MPCs is an important characteristic governing the application of these protein concentrates in food products. Fundamental research on the functional properties of proteins and the change of these properties induced by processing are thus essential to improve the MPC functionality. Emulsifying ability is one of the most important functionalities of MPC (Cameron, et al., 1991). In an emulsion, a dispersion of one liquid in another, such as oil in water (o/w), a protein acts as a surface active component forming an interface between these two immiscible liquids. This reduces the interfacial energy and therefore increases the stability of the emulsion by creating a more thermodynamically stable system (Becher, 1965).

A number of studies have shown that functionality of MPCs including solubility and emulsion capability could be related to properties of the casein (CN) micelle (Schuck & Demeler, 1999; Udabage, et al., 2012). Structural changes of the CN micelle are primarily governed by mineral equilibrium in milk and a state of the CCP. Approximately 2 /3 of the milk Ca was associated with the CCP and stabilizing the CN micelle framework (Walstra, 1990). The remaining portion is

[2] X. Luo, T. Vasiljevic, L. Ramchandran. (2015). Effect of Adjusted pH Prior to Ultrafiltration of Skim Milk on Physical Functionality of Milk Protein Concentrate. *Journal of Dairy Science*, 99(2), 1083-1094.

commonly termed soluble Ca and found in the serum phase of milk (Pierre & Brule, 1981), thus creating equilibrium between the colloidal and soluble phase (Ford, et al., 1955). Any environmental change (temperature, pH, ionic strength) in milk would lead to a shift in the casein−mineral equilibrium, resulting to an increase or decrease in concentration of free Ca ions, dissolution of the CCP from the micelle and release of specific caseins from the micelle (Holt, 1992; Gaucheron, 2005; Griffin, et al., 1988), and consequently causing the change of the micelle size. Reduction of the casein micelle size and /or Ca content appears to be plausible strategies in producing MPCs with good emulsifying properties (Ye, 2011).

Evidently a simple UF followed by a diafiltration of skim milk, while efficient in removing soluble Ca, produced MPCs with a poor solubility (Udabage, et al., 2012) or emulsifying capability (Ye, 2011). Modifications of the casein micelle appear a necessary step to achieve desired functionalities and these can be brought about by a number of approaches that would modify properties and state of the CCP including temperature (Luo, et al., 2015), pH (Dalgleish & Law, 1989; Silva, et al., 2013) or different chelators (Kaliappan & Lucey, 2011). Even the extent of concentration may change functionality of MPC (Ferrer, et al., 2011). Due to a multitude of effects it is of a great importance to establish optimum processing conditions leading to MPCs with required functionalities. UF temperature, for example, not only affected the casein micelle size but also the membrane performance with higher temperatures resulting in a smaller casein micelle size but an impaired flux and enhanced fouling (Luo, et al., 2015). Due to a profound effect of pH on the properties of the casein micelle, it would be necessary to establish an optimum pH during UF processing that would result in efficient production of functional MPCs. The CCP begins to dissociate when pH is decreased from that of normal milk (Van Hooydonk, et al., 1986). Its dissociation can induce dissociation of individual caseins (Holt, et al., 1986) and consequently release of CCP from micelles. The pH range between 5.5 and 6.7 appears critical for inducing fundamental changes of the casein micelle due to Ca^{2+} complexation with caseins (Rabiller−Baudry, et al., 2009). However the information on how these modifications would influence the functionality of MPCs as well as processing performance of UF membranes is

absent, although several predictions could be postulated as outlined above. Therefore, the aim of this study was to establish the effect of CCP solubilisation by prior milk acidification on functionality of MPC and the membrane performance. For this purpose, pH of skim milk samples, except for controls, which had 6.7, were adjusted to 6.3, 5.9 and 5.5, ultrafiltered at constant temperature (15° C) without diafiltration, freeze−dried and resulting powders assessed for selected functionalities.

6.2 Materials and Method

6.2.1 MILK SAMPLES

Skim milk, produced by a standard pasteurisation process (72 °C x 15 s) was obtained from a local supermarket (Woolworths, Melbourne, Australia) on the day of experimentation. A sodium Azide (Sigma−Aldrich Pty Ltd, Castle Hill, NSW, Australia) in the concentration of 0.02 % was added to preserve the samples for late analysis.

6.2.2 ACIDIFICATION OF SKIM MILK

As required by the experimental protocol, the sample of skim milk were adjusted to pH 6.3 5.9 or 5.5, apart from the control, which was pH 6.7, respectively, at 15 °C by addition of an appropriate volume of 0.1 and 1 M hydrochloric acid (HCl) / or 0.1 and 1M NaOH with constant stirring over a period of 60 min until the desired pH was established (Silva, et al., 2013). The total volume of all samples was kept constant by addition of an appropriate volume of milli Q−water to account for dilution caused by adding different volumes of HCl (Silva, et al., 2013).

6.2.3 PREPARATION OF MILK CONCENTRATES

All skim milk samples, the control and the pH adjusted, were maintained at a constant temperature of 15 ˚C during all UF processing using a water tank. The same size of membrane sheet, filtration system and processing parameter were used as mentioned in section 3.2.5 to produce the milk concentrates. A new

membrane sheet was used for each pH UF processing. The concentration factor was 5 (i.e. the retentate volume was 1 / 5 of the original volume).

The collected samples were measured for casein micelle size, zeta potential, total solids, and FTIR analysis immediately. The remaining samples either were frozen in −20 °C fridge or freeze dried to powder form for late analysis.

6.2.4 SAMPLE ANALYSIS

6.2.4.1 Casein micelle size and zeta potential

Casein micelle size and zeta potential of the skim milk, retentates and permeate samples were measured immediately after the collection of samples using a Malvern Zetasizer (Model ZEN3600, Nano−ZS, Malvern Instruments Ltd, UK). The method was described as previously in Section 3.3.1.

6.2.4.2 Calcium and protein content

The total and soluble Ca content of feed and retentate was determined using AAS method as described in Section 3.3.2.1. Total and soluble samples were suitably diluted to comply with a range of standard concentrations. The measured Ca content was calculated and expressed as mM. All Ca were calculated according to the standard measurements, the R^2 value = 0.98. Changes in protein conformation were observed using a Fourier transform infrared (FTIR) spectrofluorometer (Shimadzu IRAffinity−1) equipped with an IRsolution FTIR software (Shimadzu) as described previously in Section 3.3.2.2. The samples were analysed immediately after collection.

6.2.5 FUNCTIONALITY OF MPC

The MPC powder (67 % −70 % protein) was obtained by freeze drying the retentate of 5 times concentration. Physical property of MPC produced was examined within 2 weeks of powder preparation. The dispersions of MPC for the solubility and heat stability analysis were divided into two equal portions – the first which was further assessed without prior to pH adjustment and kept same as original of MPC and the second, with which pH was re−adjusted back

to the natural pH of skim milk (pH 6.7) using 0.1 or 1M NaOH. Both portions were subsequently stored at 4°C overnight to allow for full hydration of the powders. On the following day, the pH of all dispersions was confirmed and adjusted if required. The MPC emulsifying properties was assessed by the emulsion activity index (EAI) and emulsion stability index (ESI) and followed by measuring the turbidity decline and emulsion observation over 10 days (picture taken). The method for emulsion preparation and determination of other functionalities of the MPC was described in Section 3.5.

6.2.6 MEMBRANE PERFORMANCE

The membrane performance was assessed by measuring the permeate flux every 30 min during UF (perated at 15 °C) until volume concentration ratio (VCF) of 5 was achieved, and followed by an examination of membrane surface and cross section using SEM after UF operation as described in Section 3.4.

6.2.7 STATISTICAL ANALYSIS

The experiment was replicated with three sub−samplings (n = 6). The original raw data of casein micelle size was analyzed using Advanced Analytics (SAS) techniques. Other data were analysed using 1−way analysis of variance at 95 % level of confidence.

6.3 Results and Discussion

6.3.1 EFFECT OF pH ON Ca CONTENT

The Ca and phosphate distribution between serum and micellar (CN−bound) phases reported has a great influence on the functional properties of dairy products. The CCP associated with CN micelles can be varied by various treatments like heating and cooling (Holt, 1985; Luo et al., 2015), the addition of Ca−chelating agents (Udabage, et al., 2000) or acidification (Lucey & Singh, 2003).

Table 6.1. Total and soluble Ca in feed and retentates after 5 times volume concentration during UF of skim milk at 15 °C with pH adjusted to 6.3, 5.9 and 5.5.

pH	Total Ca (mM)		Soluble Ca (mM)	
	Feed, beginning	Retentate, VCF 5	Feed, beginning	Retentate, VCF 5
Control	31.08±0.81[a]	103.6±0.2[a]	12.43±0.83[a]	16.06±0.28[a]
6.3	32.23±0.41[a]	98.9±1.2[b]	15.34±0.63[b]	17.74±0.38[b]
5.9	31.40±0.71[a]	95.4±0.7[c]	17.94±1.16[b]	19.98±0.15[c]
5.5	32.71±0.78[a]	85.0±0.4[d]	25.16±1.18[c]	24.83±0.13[d]

Table 6.1 shows the total and soluble Ca in the feed and retentates after UF. pH adjustment resulted in substantial solubilisation of the CCP found in the original feed. As expected approximately 1 /3 of the total Ca was soluble (Lewis, 2011). The soluble portion increased to almost 2 /3 of the total Ca with concomitant lowering of pH (Table 6.1). While the total operating volume was reduced by 5 times, total Ca concentration increased by approximately 3.5 times indicating that colloidal phase was concentrated. Even upon concentration, a fraction of Ca was in the soluble form, which may indicate reestablishment of the mineral equilibrium. At native pH, the soluble portion of the total Ca in the retentate was 15.5 %. As the pH declined, this proportion

increased significantly from 17.9 % at pH 6.3, to 20.9 % at pH 5.9 and finally to 29.1 % at pH 5.5.

6.3.2 EFFECT OF pH ON CASEIN MICELLE AND ZETA POTENTIAL

6.3.2.1 The casein micelle size distribution

As the CCP is solubilised during acidification, an internal casein micelle structure may be affected, which is reflected in the casein micelle size distribution (Fig.6.1). The casein micelle size distribution of the casein micelles shifted initially towards smaller sizes when pH of the skim milk was changed from 6.7 to 5.9 (Fig. 6.1). No further change was observed when pH was further reduced to 5.5, being similar to casein micelle size distribution at 5.9. At natural pH of skim milk (pH 6.7), individual caseins are associated in a form of micelles, whose diameter vary depending on ionic conditions, but in general is about 200 nm in diameter.

The stability of the micelles is known to be partly due to the phosphocalcic bridge between phosphorylated lateral chains of caseins' structure and Ca (Law et al, 1998). Upon acidification, proton addition causes association of H^+ with citrate, inorganic and organic phosphates, inducing increase of ionic Ca concentration, decrease in micellar hydration and zeta potential, leading to solubilisation of micellar Ca phosphate (Law, et al, 1998). This also resulted in decline in casein micelle voluminosity (Vreeman, et al., 1989) and its casein micelle size (Silva, et al., 2013). Another contributing factor to change in casein micelle size could be dissolution of β−casein from the micelle at low temperature (Walstra, 1990). It is not clear if UF process had an impact on the decrease of the casein micelle size, although this was reported previously (Srilaorkul, et al., 1991). Studies examining mineral equilibrium and impact on the casein micelle integrity in milk have been conducted in thermodynamically closed systems, in which there was no change of overall mineral content. In our study, continuous removal of Ca^{2+} by its permeation through the membrane would presumably allow for constant and additional dissolution of the CCP. This would induce further changes of the casein micelle potentially leading to a reduction of casein micelle size. In the pH range between 5.2 and 5.9, the

casein micelle undergoes many physico−chemical changes in particular voluminosity, solvation and dissociation (Lucey & Singh, 1997; Vreeman, et al., 1989; Walstra, 1990). Accompanied by shifted equilibrium of Ca due to UF, it would be expected to obtain concentrates with a smaller casein micelle size (Srilaorkul, et al., 1991).

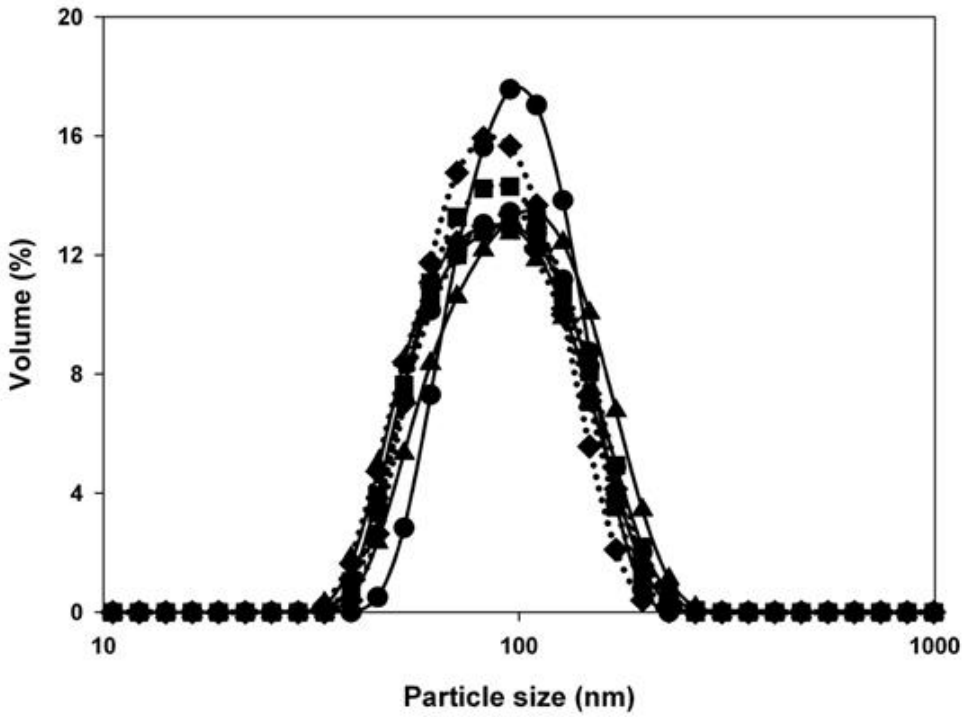


Figure 6.1. Casein micelle size distribution of feed (——) and retentate (•••••) at VCF of 5 obtained by UF of skim milk (pH 6.7 (●)) and that with adjusted pH at 6.3 (▲), 5.9 (■) or 5.5 (◆) at 15 °C.

6.3.2.2 Zeta potential

Removal of minerals, in particular Ca, by UF impacted on zeta potential (Fig. 6.2). As the feed was acidified and pH decreased from 6.7 to 5.5, the zeta potential decreased from −32.8 to −27.6 mV. On the other hand, zeta potential increased from −33.7 to −37.9 mV as the pH was decreased to 5.5.

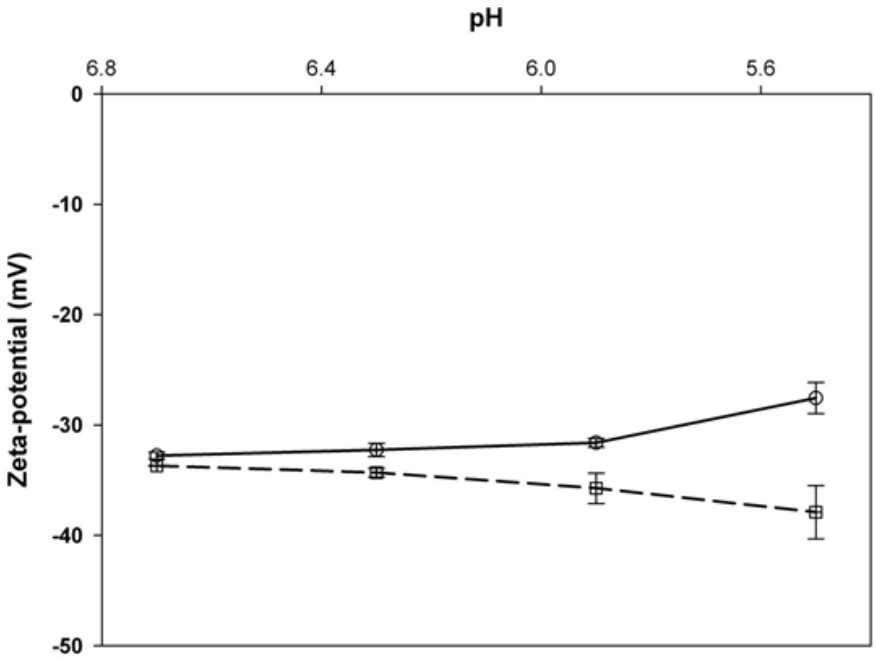


Figure 6.2. Zeta potential obtained at the beginning (—⊖—) and the end (—⊞—) of UF of skim milk at native and adjusted pH and constant processing temperature of 15 °C.

This type of observation was expected since negatively charged sites of κ−casein would become neutralized by proton addition and solubilisation of the CCP (Walstra, 1990). The exterior of the micelles mainly consists of hydrophilic negatively charged κ−casein, which provides both electrostatic and steric stabilization to the casein micelle. The negative charge is caused by dissociated carboxyl and ester phosphate group. When pH decreases, the casein−mineral equilibrium is shifted increasing the repulsion between the negatively charged amino acids in the casein micelles, leading to a decrease in concentration of free Ca ions, dissolution of CCP from micelle and release of specific caseins from the micelle (Liu & Guo, 2008). Contribution of soluble Ca to charge screening at low pH appeared substantial as Ca removal augmented negative surface potential of the particles (Fig. 6.2). This could potentially contribute to decrease in casein micelle size due to greater electrostatic repulsion along with concomitant collapse of the κ−casein hairy layer (Walstra, 1990).

6.3.3 EFFECT OF pH ON THE PROTEIN CONFORMATION

Fourier transform infrared (FTIR) spectroscope provides a wealth information about the secondary structure of proteins and is routinely used by laboratories for the analysis of conformational changes in protein of dairy products. The amide I and II region in the FTIR spectra are considered to be the region of most interest in the FTIR spectra.

FTIR interferograms (Figure 6.3A) showed an obvious change in the intensity of the peaks for the pH adjusted skim milk samples. Adjusting the pH of the skim milk to 5.9 increased the intensity of the peaks associated with Amide I, e.g. (1622 cm^{-1} (β−sheets), 1635 cm^{-1} (β−Lg), 1645 cm^{-1} (α−helixes), and 1660 cm^{-1} (turns)). These peaks are associated with β−Lg monomers in their dimeric forms (Lefevre & Subirade, 1999).

The enhanced peak intensities in skim milk of pH 5.9 indicated that at this pH, the protein had strong ordered secondary structures (e.g. β−sheets, β−Lg, α−helixes, etc.). Interestingly, with the further reduction in pH of the skim milk to 5.5, the intensity of the peaks became weaker (Fig. 6.3A). This suggests that the protein became less ordered. This is possibly due to the transition of whey protein from native towards molten globule state, as was observed by Brew and Grobler (1992) when the pH of whey protein solution was dropped to pH 5.0. This is also possibly due to the dissolution of caseins from the micelle that interacts with whey protein. The dissociation of casein is quite possible as seen from the doubling of the amount of soluble Ca in the skim milk of pH 5.5 (Table 6.1) compared to that of pH 6.7.

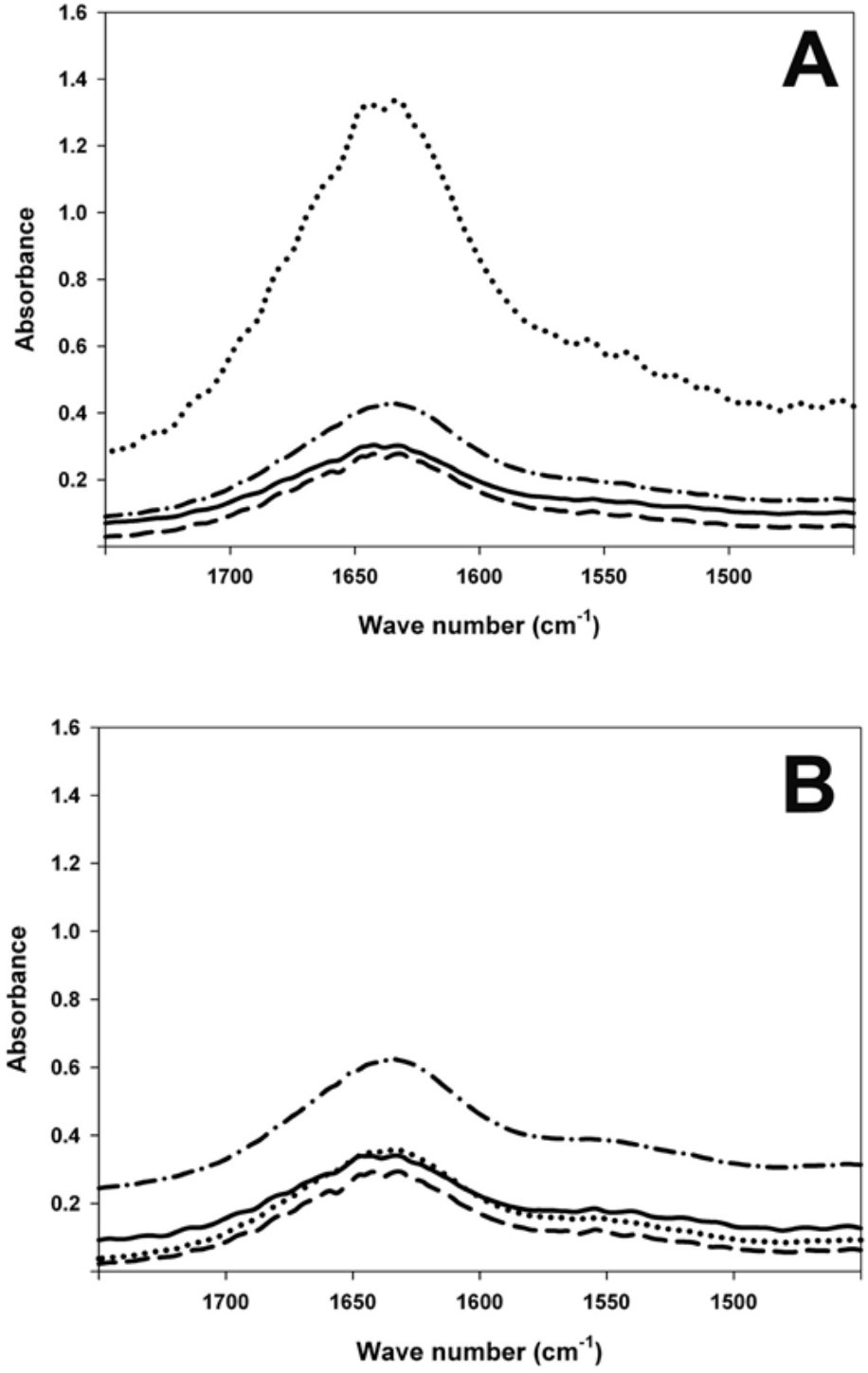


Figure 6.3. FTIR interferograms depicting structural changes of proteins upon pH adjustment at the start (A) and the end (B) of UF at 15 °C and VCF of 5. Pasteurized skim milk samples (pH 6.7) (——) and that had pH adjusted to 6.3 (— —), 5.9 (••••••) and 5.5 (—·—).

Comparing the interferograms of the pH adjusted skim milk before UF (Figure 6.3A) and that of after UF (retentate) (Figure 6.3B), it can be seen that the pattern and the peaks associated with protein structures (1622 cm^{-1} (β−sheets), 1635 cm^{-1} (β−Lg), 1645 cm^{-1} (α−helixes), and 1660 cm^{-1} (turns)) in the samples of pH 6.7 and 6.3 remained almost unchanged apart from the increase in peak intensity. There is a major change in the interferogram of the sample of pH 5.9 after UF, with that all the peaks associated with the secondary structure disappeared and accompanied by a major amplitude decrease comparing with that before UF. There is also an increase in peak intensity of the sample of pH 5.5 after UF. The increase in amplitude of the interferogram is because of the increased concentration of the protein after UF. This is in agreement with Etzion, et al. (2004) who reported that absorbance has increased when protein concentration was increased from 2 to 4 % in the milk. The amide bands were clearly visible, as is the O=P=O stretch band characteristic of casein, the amplitude of these bands increased with increasing casein protein concentration (Jung, 2000). The disappearance of the characteristic peaks associated with protein secondary structure (~ 1657 cm^{-1}) in the sample of pH 5.9 after UF implies that this sample is in similar structure as that of pH 5.5, which could be in a molten globule state.

6.3.4 THE EFFECT OF pH ON THE FUNCTIONALITY OF MPC

6.3.4.1 Solubility and heat stability

Milk proteins, due to their amphiphilic nature, play an important functional role in food. Among various functional properties, solubility is of primary importance due to its impact on the other functionalities (Halling, 1981). Numerous studies pointed out that changes in operating conditions during membrane processing impact on the composition and the functionality of the MPC powders produced (Udabage, et al., 2012). One of the limiting factors in wide spread use of MPC powders is its poor solubility, which is further compromised during storage, mainly attributed to cross-linking of casein (Havea, 2006) and migration of residual fat to the surface of the powder particle (Gaiani, et al., 2006). Figure 6.4 shows the comparison of solubility and heat stability of the reconstituted

MPC powders obtained by freeze drying of retentates of ultrafiltered skim milk that was pre−adjusted to different pH. Solubility and heat stability of the MPC dispersions (5% w/w) were also examined after neutralizing the pH of the dispersions to 6.7.

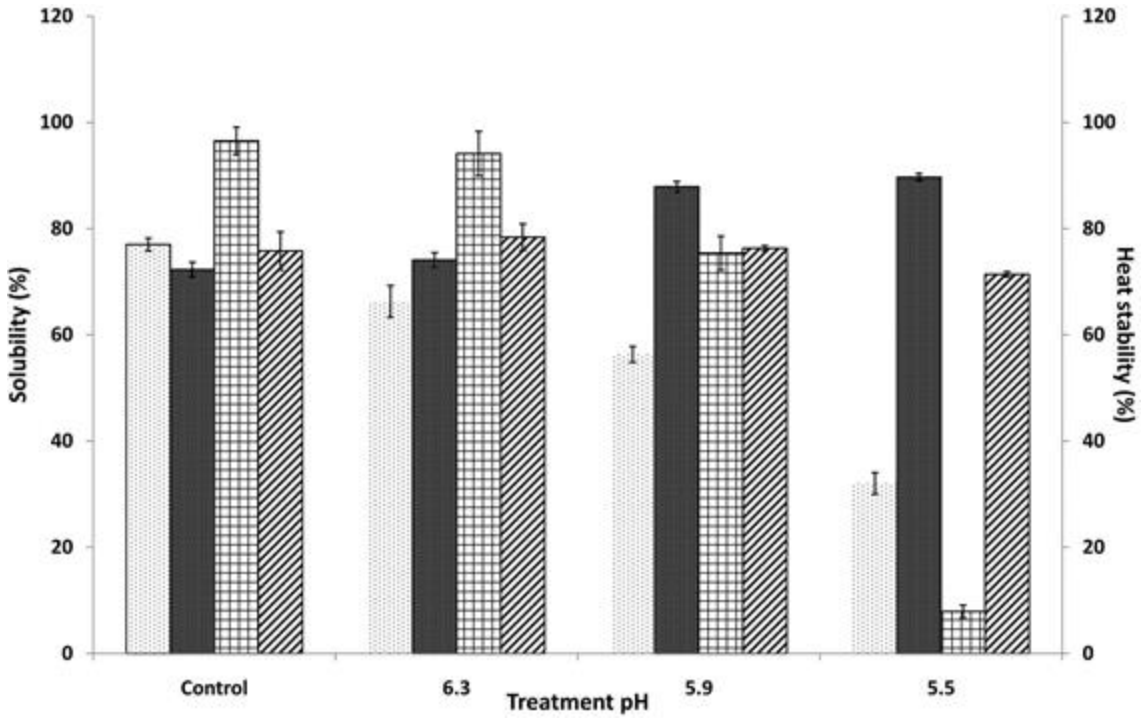


Figure 6.4. Solubility, before (⊡) and after (▩) pH adjustment, and heat stability, before (⊟) and after (◪) pH adjustment, of 5 % (w/w) dispersions prepared from MPC powders obtained by UF of pH adjusted skim milk at 15 °C.

The solubility of MPC dispersions declined substantially ($P < 0.05$) from 77 to 32 % as pH of the skim milk changed from 6.7 (control) to 5.5. However this trend reversed when the pH of the MPC dispersions was neutralized back to 6.7. The solubility of MPC dispersions increased with lower pre−adjusted pH of the skim milk prior to UF, being significantly ($P < 0.05$) higher for that of pH 5.9 (87.9 ± 1.0) and 5.5 (89.7 ± 0.7) than that of the control (77.0 ± 1.0). The heat stability followed a similar trend, which was declining from 96 to 8 % for the pH of skim milk pre−adjusted from pH 6.7 to 5.5 prior to UF. Neutralizing pH of the MPC dispersions once again improved heat stability, which was comparable among the samples ranging between 72 and 78 % (Figure 6.4). Among various

factors influencing protein solubility, pH plays a central and important role. At pH above and below their isoelectric point (pI), proteins have a net negative or net positive charge, respectively, which contributes to overall protein stability and consequently solubility. At pH closer to isoelectric point, casein micelle starts to change due to collapse of κ−casein hairy layer (Walstra, 1990) and solubilisation of the CCP resulting in compromised steric hindrance and lower electrostatic repulsions due to released Ca^{2+} (Table 6.1). Accompanied with lower processing temperature as in our study, β−casein dissolves and leaks out of the micelle into the surrounding medium due to minimized hydrophobic attractions (Kehoe & Foegeding, 2011), leading to a system with substantially different physical properties upon drying and redispersion. Modified surface properties likely resulted in poor dispersability and enhanced aggregation, which was manifested by poor solubility. Additionally, released β−casein might have been located on the surface of particles upon water removal, which further enhanced hydrophobic interactions upon reintroduction into solution driving particles towards aggregation and poor solubilisation. These interactions however appeared minimized upon neutralisation, which have likely recovered some of the initial surface properties.

Differences in the heat stability of milk have been attributed to variations in the composition of milk salts and presence or absence of the CCP (O'Connell & Fox, 2003). For example, Ghatak, et al. (1989) reported that the heat stability of both bovine and buffalo milk is significantly correlated to soluble Ca content while Pouliot & Boulet, (1995) correlated the heat stability of concentrated milk to the ratio of colloidal Ca to colloidal phosphate. Furthermore, a mode of milk protein concentration has a marked effect on heat stability. Concentrates prepared by UF were more stable than those produced by conventional evaporation, which was attributed to a reduction in the concentration of lactose and soluble Ca (Sweetsur & Muir, 1980). Lowering pH beyond 6.0 prior to UF resulted in concentrates with compromised heat stability (Muir & Sweetsur, 1984), once again indicating substantial changes of surface properties invariably leading to low solubility and heat stability. Apparently some of these physicochemical changes may be reversed by pH readjustment upon redispersion thus improving solubility and heat stability.

6.3.4.2 The emulsion activity and emulsion stability

Modified particle properties may ultimately lead to improved or compromised interfacial functionality. The emulsion activity index (EAI) and emulsion stability index (ESI) of reconstituted MPC powders obtained by pH adjustment are presented in Figure 6.5. Lowering pH to 5.9 improved ($P < 0.05$) EAI; however it consequently declined ($P < 0.05$) at pH 5.5. This trend was conversely reversed upon neutralization with EAI at pH 5.5 being similar ($P > 0.05$) to that at pH 5.9 and greater than EAI at pH 6.7 or 6.3 (Figure 6.5). The emulsion stability as indicated by ESI on the contrary was greater ($P < 0.05$) at the control pH and steadily declined ($P < 0.05$) as pH was lowered prior to UF. Interestingly neutralisation completely reversed this trend, with the most stable emulsion obtained using MPC obtained at pH 5.9, being almost unchanged during the time of experimentation.

Emulsion activity index is a measure of the capability of a protein to stabilise the interface between two immiscible phases, such as oil and water (Dickinson, et al., 1992; Dalgleish, 2006). Stability of an emulsion depends on the balance of several forces including attractive, repulsive, steric and depletion forces (Dickinson, 1997; Chiralt, 2009). The food emulsions are very complex in composition and structure, the structure of an emulsion can change in several ways including creaming, flocculation, or coalescence or their combinations (Dickinson, 1997). Thus emulsion stabilisation may be provided by hindering these processes manipulating forces driving them. For an emulsion to remain kinetically stable, repulsive forces need to be greater than attractive forces. From Figure 6.5, it is obvious that MPC powders had different emulsification properties likely governed by surface properties of constituting particles. Dispersions prepared by MPCs without pH adjustment apparently had compromised solubility that consequently impacted on redistribution of protein particles on the interface and thus emulsifying capacity. Upon neutralization, many amphiphilic properties were restored and probably improved since MPCs obtained at pH 5.9 and 5.5 had greater EAI and ESI than those obtained by UF at 6.7 or 6.3 (Figure 6.5). Such an improvement could partially be attributed to reduced casein micelle size, although it does not appear as a governing factor at lower pH since MPCs obtained at 5.5 and 5.9 had similar casein micelle size

distributions (Figure 6.1). Loss of steric hindrance and hydrophilicity by collapsing κ−casein (O'Connell & Fox, 2003) might have rendered resulting casein micelles more hydrophobic and prone to association thus poor solubility. This however upon pH neutralisation might have been counteracted by increased electrostatic repulsion due to lack of soluble Ca in solution thus improved solubility. However, rather looser structure due to depletion of the CCP at low pH and likely reorganisation of individual caseins (Anema & Klostermeyer, 1997; Vasbinder, et al., 2003) resulted in smaller particles with enhanced emulsifying properties. While it is not evident from the current study, dissolution of β−casein during UF at low temperature is expected; however it is difficult to predict its fate during further processing and whether it plays any role in improved emulsification. The β−casein is highly hydrophobic and prone to self−associations creating different micellar forms and modifying behaviour of other present proteins (Liyanaarachchi, et al., 2015).

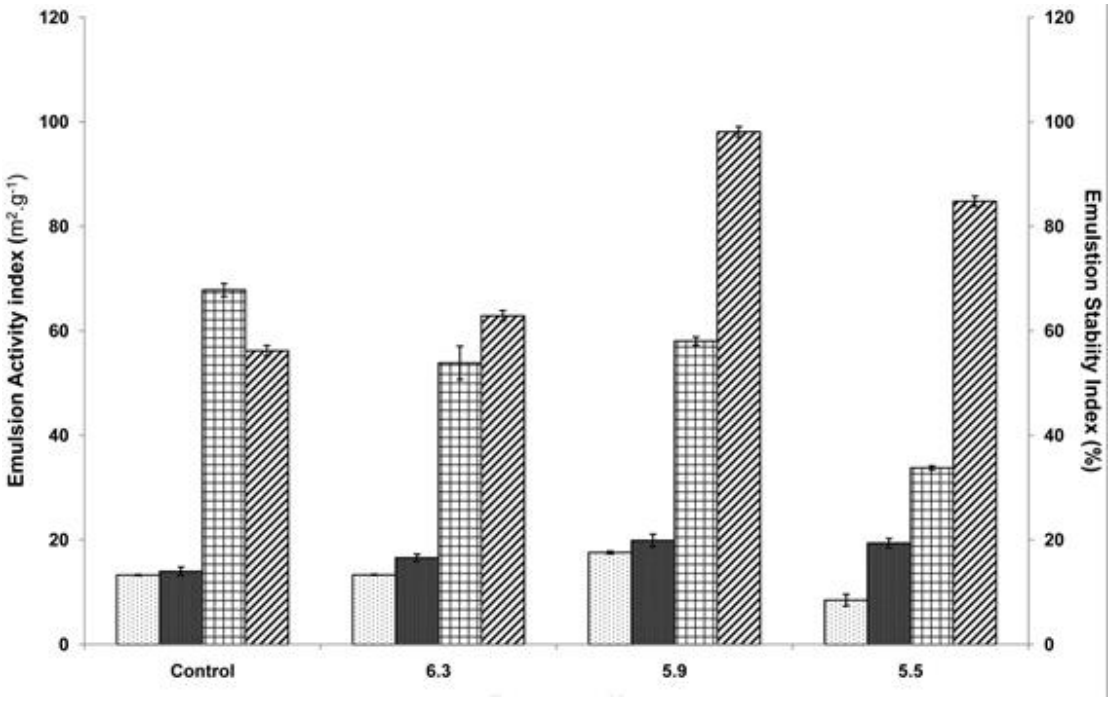


Figure 6.5. Emulsion activity index, before (⊡) and after (▩) pH adjustment, and emulsion stability index, before (⊞) and after (◪) pH adjustment, of emulsions prepared from MPC powders obtained by UF of pH adjusted skim milk at 15 °C.

6.3.5 EFFECT OF pH ON THE MEMBRANE PERFORMANCE

Achieving a balance between process parameters to obtain MPC of required functionality and membrane performance is of paramount importance. The membrane performance was initially assessed by flux determination and further elaborated on by the SEM characterisation. As expected the flux declined steadily over time during UF irrespective of adjusted pH (Figure 6.6). The flux was similar ($P > 0.05$) for all UF runs for the skim milk of pH 6.7, 6.3 and 5.9. The flux decreased substantially ($P < 0.05$) as pH of the skim milk was adjusted to 5.5. It took approximately 3.5 h to achieve VCF of 5 at pH 5.9 or higher, while this time was extended to 4 h at pH 5.5. Apparently, pH adjusted to less than 5.9, it induced significant changes in surface properties of the casein micelle that resulted in enhanced surface activity and subsequent greater fouling at lower pH. This membrane fouling was also observed by SEM (Figure 6.7). The cross section of the membrane operated at pH 6.7 appeared clean as indicated by the presence of some clear pores on the surface and rigid supporting wall. As pH was lowered to 5.5, the extent of membrane fouling became substantial, with pores and supporting wall being almost completely blocked at pH 5.5 (Figure 6.7).

Membrane fouling in the dairy industry mainly arises from the composition and properties of the treated fluid apart from the effects of operation parameters (e.g., pressure, temperature and flow rate). Membranes employed in UF of milk are usually hydrophilic, which minimizes attraction between membrane surface and proteins. Therefore, creation of a fouling layer is initiated by soluble salts, mainly Ca and phosphates (Hausmann, et al., 2013). Salt deposition may be accelerated at lower pH due to greater salt solubilisation from the casein micelle. Upon layer creation, these salts start interacting with the casein micelles, which modified structure allows for greater attraction and consequently greater deposition and fouling. However while the fouling layer appeared thicker at low pH, the rate of flux decline among all runs was similar, which may be indicative of a highly porous layer (Hausmann, et al., 2013). Similarly Patel & Reuter (1985) attributed flux impairment during UF processing at lowered pH to increased concentration of soluble Ca, which is in agreement with our finding (Table 6.1). Additionally, the low pH is always accompanied by

an increase in the viscosity of milk, which may further hinder the flux. All these factors have been implicated in fouling of membranes and questionable process feasibility (Muir & Sweetsur, 1984).

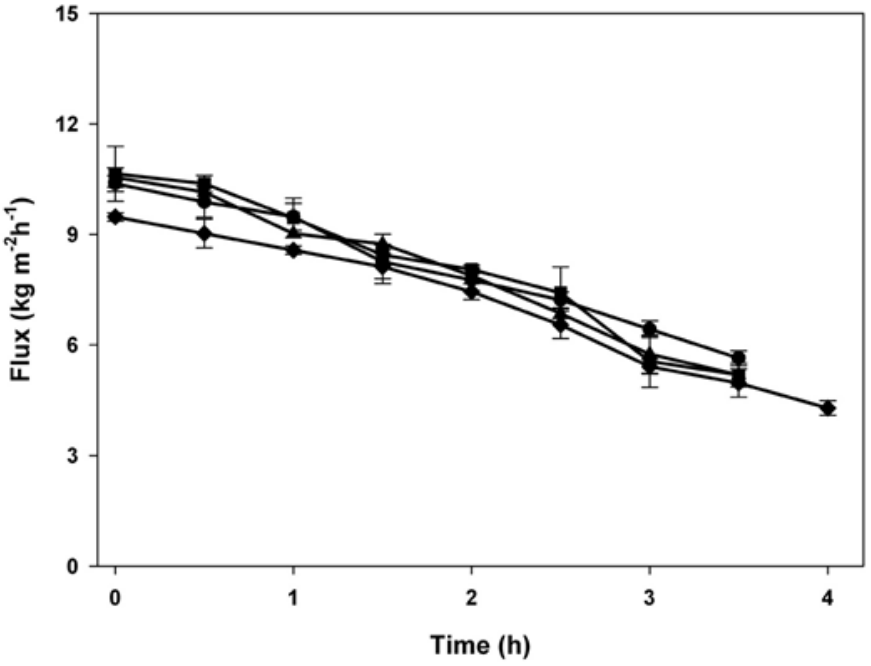


Figure 6.6. Permeate flux during UF of skim milk (pH 6.7 (●)) and that with adjusted pH at 6.3 (▲), 5.9 (■) or 5.5 (◆) at 15 °C to a VCF of 5.

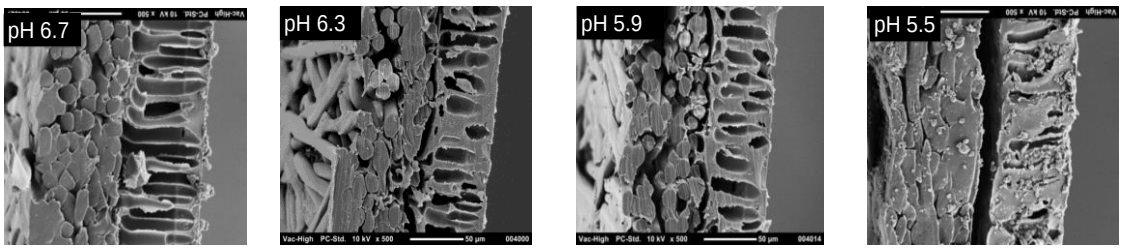


Figure 6.7. Scanning electron microscopy (SEM) obtained cross sections of membranes at the end of UF of skim milk (pH 6.7) and that with adjusted pH at 6.3, 5.9 or 5.5 at 15 °C and a VCF of 5.

6.4 Conclusion

Soluble Ca increases with the decrease of pH of skim milk after skim milk was concentrated may be induced by the acidification, resulting in the casein micelle size reduced when decrease in pH of skim milk during the UF processing.

Pre−adjust the pH of skim milk from 6.7 to 5.5 made the protein structure appears disordered and further disordered when 5 time concentration was achieved (at pH 5.5). Several reasons could be involved for the protein deformation. Such as the viscosity increased when skim milk was concentrated; the milk Ca was reduced and re−arranged after the acidification and followed UF processing, consequently, the casein micelles changed in a dynamic processing.

Pre−adjustment of the pH of the skim milk from 6.7 to 5.9 prior to UF yielded MPCs with enhanced functionalities (solubility, heat stability, emulsion activity and emulsion stability).

However, further lowering of the pH of the skim milk to 5.5, some of the MPC functionalities (e.g. heat stability and emulsion stability) started to decline, and also membrane fouling has increased.

To achieve the balance of obtaining MPC with enhanced functionality and less membrane fouling, the best pH of the skim milk prior to UF is 5.9 as assessed from this study.

Chapter 7. **Effect of Chelators on Emulsification Properties of MPCs at a Constant pH and Temperature of UF**

7.1 Introduction

MPC are usually produced by membrane filtration such as UF or UF / DF. MPC can offer a great potential for use in an array of food applications because of their nutritional and functional values. However, MPC powders with protein content greater than 80 % exhibit poor solubility (Sikand, et al., 2013; Huppertz, 2010). Additionally the size of micelles in MPC may influence their functionalities such as emulsifying properties when MPC is used in dairy products (Ye, 2011).

The casein, as a major protein in milk, appears in the form of intact casein micelles in milk concentrates and is naturally present as a colloidal, poly-disperse, and spherical particle with an average diameter of ~200 nm (Holt, 1992), containing about 7 g minerals per 100 g dry casein, which has been termed CCP. The exact composition of CCP depends on the ionic environment, which means that it has ion−exchange properties (Gaucheron, 2005; Walstra, et al., 2006). Dissociation of CCP can be achieved as a result of acidification, cooling or addition of chelating agents to milk accompanied by release of individual caseins from the casein micelle (Rose, 1968; Lin, et al., 1972; Holt, et al., 1986; Dalgleish & Law, 1988; 1989).

Calcium chelators such as citrate, phosphate, hexametaphosphate (HMP), or EDTA are commonly used in the dairy industry to improve the heat stability or to retard age gelation in dairy products (Augustin & Clarke, 1990; Harwalkar, 1982; Holt, 1985; Singh, et al., 1995). This is achieved by shifting protein−mineral equilibrium, leading to a decrease in the concentration of free Ca ions, depletion of CCP from the micelles and release of specific caseins from the micelle (Holt, 1992; Gaucheron, 2005; Panouille, et al., 2004), which finally induces various physical changes in the casein micelle.

The release of minerals from the casein micelles to the serum was reported to increase the repulsion between the negatively charged amino acids in the casein micelles, resulting in an increase in hydration and voluminosity of the micelles (Gaucheron, 2005; Walstra, 1979), decrease in turbidity of milk (Walstra, 1990; Panouille, et al., 2004), and increase in viscosity through interactions with the casein proteins when Ca chelators were added. The micelles eventually dissociate into small clusters and dispersed proteins at higher chelator levels.

The extent to which chelators affect the micellar structure depends on their Ca−binding capacity (De Kort, et al., 2009) and their interaction with the Ca ions and amino acids in the casein micelle (Mizuno & Lucey, 2007). Besides the generic property of Ca−binding, Ca chelators may generate specific effects. Studies by Panouillé, et al. (2005) and Pitkowski, et al. (2008) have shown that intact and dissociated casein micelles can be present simultaneously in the milk after addition of chelators. Kaliappan and Lucey (2011) attempted to modify physical properties of milk proteins such as heat stability by addition of phosphates and citrates to milk system. Sodium salts of phosphoric or citrates were added to increase the heat stability of concentrated or sterilized milk (Sweetsur & Muir, 1980) and to decrease the susceptibility of ultra−high temperature processed milk products to age−induced gelation (Walstra, 1999). Udabage, et al. (2000; 2001) found that the EDTA, citrate, and other chelators at pH 6.65 affected the size of casein micelles, and the addition of citrate or EDTA, which removed more than 33 % of the original CCP with accompanying release of 20 % casein from the micelle, completely inhibited gelation.

The use of EDTA is frequent as a chelator due to its well-known effect on dissociation of casein micelles (Lin, et al., 1972). The effect of EDTA on the physical changes of the casein micelle in skim milk has been extensively studied (Lin, et al., 1972; Griffin, et al., 1988; Ward, et al., 1997; Udabage, et al., 2000). Information is also available on the effects of phosphates and citrate on the voluminosity of the casein micelle and turbidity of the milk (Leviton & Pallansch, 1962; Vujicic, et al., 1968; Mizuno & Lucey, 2005). The effect of citrate on physical changes of milk has been studied in skim milk systems, with low concentration factors (protein ~6.5 %, w/v), and relatively low chelator

levels and mainly focused on the formation of milk gels (Mizuno & Lucey, 2005) or on age gelation after addition of chelators (Kocak & Zadow, 1985).

Previous research has investigated the effect of Ca chelators on dairy products to improve heat stability and gelation. However, the solubility and other functionalities regarding the change on the casein micelles size induced by shift of protein-mineral equilibrium have not yet been reported. The information on whether further shift induced by Ca^{2+} partially permeated/removal before or during UF processing is also limited especially the extent to which they are affected by different concentrations of EDTA and citric acid.

The aim of this study was to understand the physicochemical properties and functionalities of the MPC produced as well as the performance of membrane during UF of skim milk with added Ca chelating agents.

7.2 Materials and Method

7.2.1 MILK FEED PREPARATION

The skim milk used for this study was provided by a local supplier (Woolworths, Melbourne, Australia), and pasteurised using the traditional method (72 °C x 15 s). Temperature (15 °C) and pH (5.9) of skim milk were pre−set based on the results obtained in the previous chapters (Chapter 5 for temperature and Chapter 6 for pH). Three levels (10, 20 or 30 mM) of the chelators were prepared using EDTA (200 mM of EDTA, MW = 372.3) or citrate (200 mM Tri−sodium citrate, MW = 294.10). The skim milk was pre-treated with the chelator solutions thus prepared by slowly adding to the skim milk to the final concentration required while stirring at 15 °C and final pH of the milk was adjusted to pH 5.9 using 0.1 or 1 M HCl. The effect of dilution due to addition of chelator was tested and found to be negligible ($P < 0.05$) as small amount of concentrated chelator (EDTA or citrate) and HCl were added to 1 L of skim milk.

7.2.2 PREPARATION OF MILK CONCENTRATES

All skim milk samples, the control and that with addition of EDTA or citrate,

were maintained at 15 ˚C during UF process using a water tank. The same membrane unit and processing parameter were used as described in section 3.2.5 to produce the milk concentrates. A new membrane sheet was used for each UF process. The concentration factor was 5 (i.e. the retentate volume was 1 / 5 of the original volume).

7.2.3 SAMPLE ANALYSIS

The sample treated with 10, 20 and 30 mM of EDTA or citrate has been designated as E10, E20 and E30 or C10, C20 and C30 respectively. The collected samples were measured for casein micelle size, zeta potential, and FTIR analysis immediately. The remaining samples were either frozen in −20 °C freezer or freeze dried for analysis.

7.2.3.1 Casein micelle size and zeta potential

Casein micelle size and zeta potential of the skim milk, retentates and permeates were measured immediately after the collection of samples using a Malvern Zetasizer (Model ZEN3600, Nano−ZS, Malvern Instruments Ltd, UK). The method has been described in section 3.3.1.

7.2.3.2 Calcium content

The total, soluble and ionic Ca content of the control and chelator treated samples before and after UF were analysed using the methods as described in section 3.3.2.1. The control and chelator treated samples before and after UF were diluted to comply with a range of standard concentrations from 1 − 10 ppm. The measured Ca content was calculated and expressed as mM. All Ca were calculated according to the standard measurements, $R^2 = 0.98$.

7.2.3.3 Protein conformation

Changes in protein conformation were observed using a Fourier transform infrared (FTIR) spectrofluorometer (Shimadzu IRAffinity−1) equipped with an IRsolution FTIR software (Shimadzu) as described previously in section 3.3.2.2. The samples were analysed immediately after collection.

7.2.4 FUNCTIONALITY OF MPC

The functionality of MPCs was examined within 10 days of powder preparation. The freeze dried MPC powder samples were stored in -20 °C freezer inside sealed yellow top plastic containers. The dispersions of MPC (5 % w/w) for the solubility and heat stability analysis were produced and stored at 4 °C overnight to allow for full hydration of the powders. The MPC emulsifying properties was assessed by the emulsion capacity (EC) and emulsion stability (ES) and followed by measuring the turbidity and visual observation for 10 days. The method for determination of solubility, heat stability and emulsion properties of the MPC was the same as described in section 3.5.

7.2.5 MEMBRANE PERFORMANCE

The membrane performance was assessed by measuring the permeate flux every 30 min during UF until VCF of 5 was achieved, and followed by an examination of membrane surface and cross section using SEM after UF operation as described in section 3.4.

7.2.6 STATISTICAL ANALYSIS

The experiment was replicated with three sub−samplings (n = 6). The original raw data of casein micelle size was analysed using Advanced Analytics (SAS) techniques. Rest data were analysed using 1−way analysis of variance at 98 % level of confidence.

7.3 Results and Discussion

7.3.1 EFFECT OF CA CHELATORS ON CASEIN MICELLES (CASEIN MICELLE SIZE AND ZETA POTENTIAL)

7.3.1.1 Casein micelle size

The casein micelle size distribution (Figure 7.1) of the control (without addition of chelator) before (the skim milk) and after UF concentration of 5 times were similar. The casein micelle size for both samples ranged between 20 and 200 nm and without apparent aggregation. The distribution of casein micelle size was slightly different from that of De Kruif and Holt (2003) who reported that casein micelle size ranged from 80 to 550 nm with an average of about 200 nm.

When 10 mM EDTA was added to skim milk prior to processing, two small peaks appeared, one on the left hand side with a size range of 10 − 20 nm and the other on the right hand side with size range of 2000 − 4000 nm. This indicated that the addition of 10 mM EDTA caused dissociation of some casein micelles and some aggregation. After UF, the two extra peaks disappeared and the casein micelle size distribution became wider than that of the corresponding control sample (Figure 7.1).

Upon further addition of EDTA to 20 mM, a small peak appeared in the range of 3 − 10 nm. The original small peak of 10 − 20 nm size became the major peak whereas the peak at 20 − 200 nm became significantly smaller. The size range between 2000 - 4000 nm didn’t change. This indicated that with the addition of EDTA to 20 mM level, the casein micelle size of most casein micelles reduced which was induced by dissociation of casein micelles. The dissociation of casein micelles could be caused by the binding of Ca with chelators that disturbed the equilibrium between the Ca and casein in the micelles. After UF, the major peak at 10 - 20 nm became stronger and the shoulder at 2 − 10 nm became a separate peak (2 − 9 nm). This indicated that UF had further reduced casein micelle size.

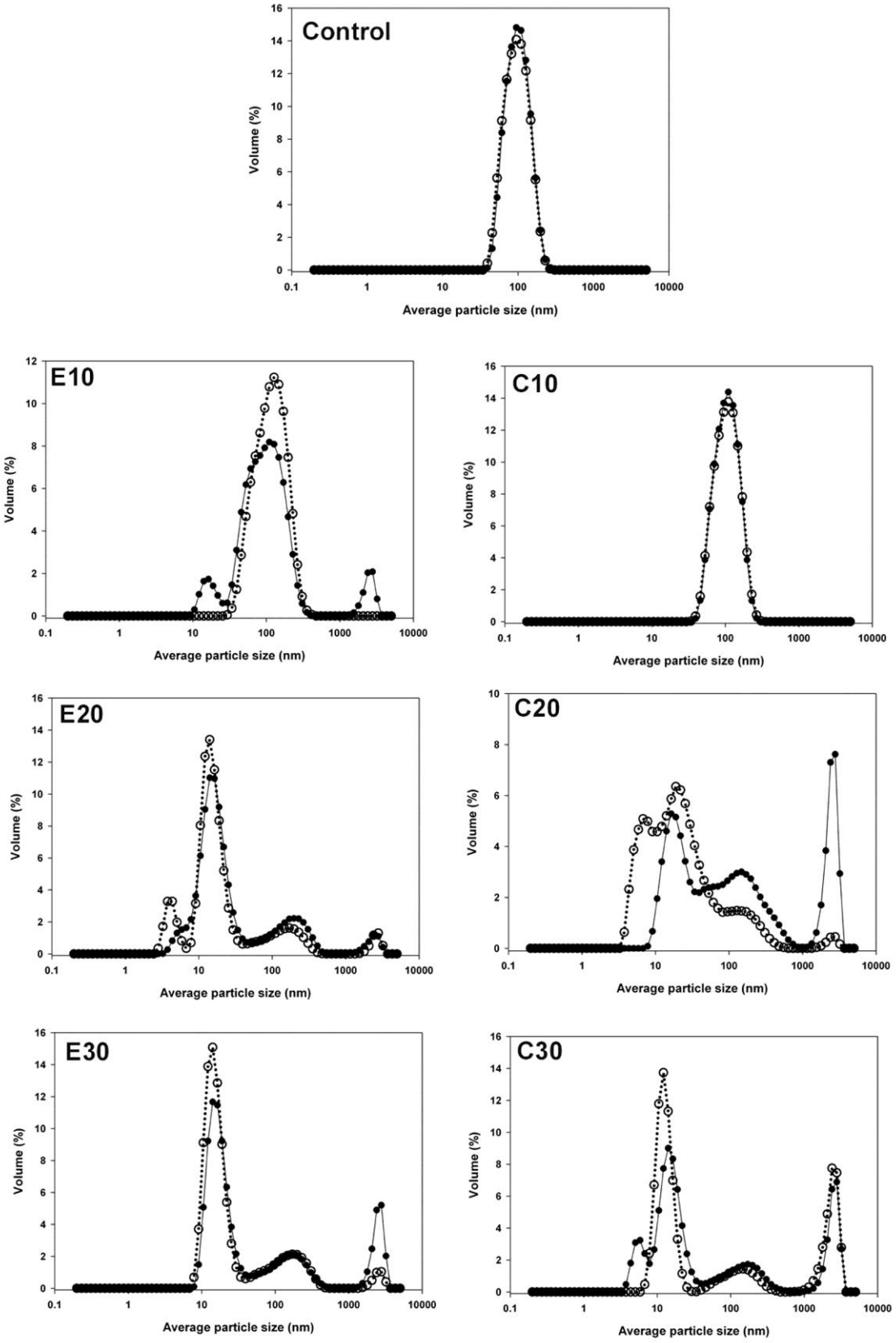


Figure 7.1. The casein micelle size distribution of retentates obtained at the beginning (—●—) and the end (··⊙··) of UF with samples treated with 10, 20 and 30 mM of EDTA or citrate.

Upon further addition of EDTA to 30 mM, the casein micelle size distribution is similar to that of 20 mM of EDTA added sample apart from the peak at 2000 − 5000 nm that became more pronounced. This indicated that more aggregation occurred when EDTA concentration was increased. After UF, the peak at 9 − 20 nm became stronger and that at 2000 − 4000 nm became smaller. This indicated that the UF had broken some of the aggregates.

Certain substantial differences in casein micelle size distribution patterns between EDTA and citrate treated samples were observed (Figure 7.1). The casein micelle size distribution of 10 mM citrate treated sample was almost identical to that of the control and remained basically unchanged after UF. Upon further addition of citrate to 20 mM, a substantial change was noted with the appearance of a major peak at range of 10 − 20 nm and another peak at 1000 − 3000 nm. After UF, the casein micelle size distribution shifted to the left with a new peak at 3 − 10 nm appearing, overlapping with the original but stronger peak at 10 − 70 nm. The original peak at 70 − 900 nm became weaker and the peak at 1000 − 3000 nm almost disappeared. With the further addition of citrate to 30 mM, two major peaks at 10 − 20 nm and 1000 − 3000 nm remained strong, and the peak at 20 − 200 nm became a small one with the appearance of another small peak at 3 − 10 nm. This indicated that the addition of 30 mM citrate caused further dissociation of casein micelles and some small protein particles in the size range of 3 − 10 nm appeared. After UF, the small peak at 3 − 10 nm disappeared and the peak at 8 − 20 nm became the major peak and the other two peaks at 20 − 200 nm and 1000 − 3000 nm remain unchanged. This indicated that UF caused aggregation of small particles (3 − 10 nm) and increased the population of the particles in the range of 8 − 20 nm for the samples with the addition of 30 mM citrate.

It is generally accepted that caseins with Ca phosphate are assembled into casein micelles (Muller−Buschbaum, et al., 2007). When a Ca chelator is added to the milk system, the casein-mineral equilibrium is shifted. This shift increases the repulsion between negatively charged amino-acid in casein micelles, resulting in change in zeta potential, leading to a dissolution of CCP from micelle and release of specific caseins from the micelle (Holt, 1992; Gaucheron, 2005; Panouille, et al., 2004). Casein micelles eventually dissociate into smaller

clusters upon addition of chelators (Panouille, et al., 2004; Marchin, et al., 2007; Griffin, et al., 1988; Udabage, et al., 2000) because the integrity and stability of the supramolecular structure of CMs are strongly associated with their mineral content, especially CCP, when Ca−chelating agents (e.g. EDTA or citrate in this study) were added to milk or micellar suspension, the solubilisation of CCP induced dissociation of CMs (Lin, et al., 1972; Holt, 2004) leading to the reduction in casein micelle size.

The amount of EDTA and citrate required to induce casein micelle size reduction is different. A significant casein micelle size reduction occurred when ≥ 20 mM of EDTA was added to skim milk, whereas for citrate, ≥ 30 mM is required (Figure 7.1). EDTA could be used at lower concentrations than citrate because of its higher Ca ion binding capacity. This is in agreement with the observations of Udabage, et al. (2003) and Keowmaneechai and McClements (2002).

7.3.1.2 Zeta potential

The zeta potential (Table 7.1) increased with the addition of EDTA or citrate acid and further increased after UF. The zeta potential of the skim milk (control) before UF was −31.1 mV and it became −33.2 mV after UF.

Addition of 10, 20 or 30 mM of EDTA resulted in an increase ($P < 0.05$) in zeta potential to −33.9, −37.1 or −39.5 mV, respectively. After UF, the zeta potential changed to −37.5, −42.22 or −42.2 mV respectively. The difference in zeta potential between that of control and that of E10 is non-significant ($P > 0.05$) but between that of control and that of E20 or E30 is significant ($P < 0.05$).

The zeta potential of skim milk with addition of 10, 20 or 30 mM citrate is −35.6, −32.8 or −32.3 mV, respectively which went up to −39, −36.8 or −35.8 mV, respectively after UF. The difference of zeta potential between that of control and that of citrate added is only significant ($P < 0.05$) for the 10 mM citrate sample after UF.

The impact of different types of Ca chelators on the mineral equilibrium and casein micelle structure can vary due to differences in affinity of chelators to Ca ions and the different interaction of chelators with the amino acid residues of

caseins (Punsandani, et al., 2000; 2001). Therefore, the zeta potential of EDTA and citrate treated samples are different. It is difficult to compare zeta potential in this study with other reports due to differences in experimental conditions, such as diluent used and temperature, which greatly influenced the values obtained (Philippe, et al., 2005). In this study, addition of EDTA or citrate showed an absolute increase in zeta potential with rise in chelator concentration. After UF, the zeta potential further increased in absolute terms for either EDTA or citrate treated samples. This was in agreement with Hunter (1988) who reported an increase in zeta potential when the raw skim milk concentration was increased, which may be attributed to the reduced water mobility with the closer packing of particles in retentates after 5 times concentration using UF.

7.3.2 EFFECT OF Ca CHELATORS ON Ca DISTRIBUTION

The total Ca in cow milk is about 29.4 mM, out of which the soluble Ca is 9.2 mM and micellar Ca is 20.2 mM (Holt & Jenness, 1984). The total, soluble and ionic Ca contents in the retentate of skim milk as well as those with added Ca chelators are shown in Table 7.1, both before and after UF. Because of concentration by UF, total Ca increased significantly ($p < 0.05$) from 28−30 mM (feeds) to 74−89 mM (retentates) and soluble Ca also increased significantly ($p < 0.05$) from 10−16 mM (feeds) to 57−70 mM (retentates).

The total Ca of untreated control was 29 mM in the skim milk before UF and became 87.8 mM after UF (retentate control). With addition of EDTA from 10 to 30 mM, there was no significant change in the total Ca before UF, however, after UF, the total Ca reduced from 88.9 (for E10) to 80 mM (for E30) in retentates. There were also no significant changes in total Ca in the retentate of the 10 mM of EDTA or citrate added samples compared to that of the control. However, there was a significant difference in total Ca in the retentate (after UF) for the 20 or 30 mM of EDTA or citrate added samples compared to that of control. It decreased from 87.8 mM to 80.0 or 80.4 mM for the addition of 20 or 30 mM of EDTA, and to 78.4 or 73.9 mM for the addition of 20 or 30 mM of citrate.

Table 7.1. Concentration of the total, soluble and ionic Ca and related zeta-potential of the samples obtained at the beginning and the end (VCF 5) of UF of skim milk treated with 10, 20 and 30 mM of EDTA or citrate at adjusted pH 5.9 and constant UF temperature of 15 °C.

Chelator	Total Ca (mM)		Soluble Ca (mM)		Ionic Ca (mM)		Zeta-potential (mV)	
	Beginning	End	Beginning	End	Beginning	End	Beginning	End
Control	29.0^{a}	87.8^{a}	10.3^{a}	63.1^{b}	2.46^{a}	4.04^{a}	-31.1^{a}	-33.2^{a}
E10	29.6^{a}	88.9^{a}	10.1^{a}	62.6^{b}	1.64^{b}	3.07^{b}	-33.9^{a}	-37.5^{ab}
E20	30.0^{a}	80.0^{b}	13.4^{b}	69.1^{c}	1.04^{c}	1.91^{d}	-37.1^{bc}	-42.2^{cd}
E30	28.9^{a}	80.4^{b}	13.2^{b}	62.1^{b}	0^{e}	0^{g}	-39.5^{c}	-45.3^{d}
C10	28.3^{a}	87.9^{a}	11.4^{ab}	56.6^{a}	1.54^{b}	2.70^{c}	-35.6^{ab}	-39.0^{bc}
C20	28.1^{a}	78.3^{b}	14.0^{bc}	64.7^{b}	1.11^{c}	0.96^{e}	-32.8^{a}	-36.8^{ab}
C30	27.7^{a}	73.9^{c}	15.9^{c}	70.1^{c}	0.55^{d}	0.28^{f}	-32.4^{a}	-35.8^{ab}
SEM	1.2		1.5		0.05		2.4	

Soluble Ca in the untreated control sample was 10.3 mM and increased ($P < 0.05$) after UF (retentate control) to 63.1 mM due to the concentration effect of UF. Comparing with the corresponding control, the addition of 10 mM EDTA or citrate had no apparent impact on the soluble Ca content in the skim milk before UF (10.1 mM vs 10.3 mM (untreated control)) and after UF (62.6 mM vs 63.1 mM (retentate control)). Addition of 20 mM of EDTA resulted in a significant increase ($P < 0.05$) in soluble Ca to 13.4 mM (before UF) and 69.1 mM (after UF). With addition of 30 mM EDTA, the soluble Ca content remained the same as that of 20 mM sample before UF, but that after UF was lower (62.1 mM). Similarly, addition of 20 or 30 mM of citrate resulted in a significant increase in soluble Ca to 14.0 and 15.9 mM, respectively, before UF. It is well known that the Ca distribution between soluble and insoluble phases is greatly influenced by the addition of Ca−chelating agents (Odagiri & Nickerson, 1964; Udabage, et al., 2000). Ca chelating agents have a high affinity for cations and are able to bind Ca (usually in a one to one ratio) through carboxylic groups, and are able to displace Ca from casein micelle (Brulé & Fauquant, 1981). Thus, the addition Ca−chelating agents to milk induced an increase in soluble Ca in the milk (Table 7.1). The increased concentration of the soluble Ca in the

serum when more EDTA or citrate was added was due to a shift in the casein-mineral equilibrium caused by removal of Ca from the Ca−protein complexes in the casein micelle (Patocka & Jelen, 1987). Lowering of pH due to addition of EDTA could further have contributed to the increase in soluble Ca (Chandrapala, et al., 2010a, b).

The ionic Ca of the skim milk (control) was 2.46 mM before UF which increased to 4.04 mM after UF. Upon addition of chelators, EDTA or citrate, the concentration of ionic Ca decreased substantially indicating complexation of ionic Ca resulting in lower ionic Ca (Table 7.1). When Ca chelator is added, it binds to the Ca^{2+} via carboxylic groups leading to a decrease in the concentration of free Ca ions (De Kort, et al., 2012).

EDTA and citrate act slightly differently as chelators and showed variations in the extent of increase in soluble Ca and decrease in ionic Ca (Table 7.1) because chelators have different affinity for Ca ions. This gives them varying ability to release CCP from the casein micelles (De Kort, et al., 2009; Upreti, et al., 2006; Mekmene, et al., 2009). For example, the soluble Ca was 13.4 or 14.0 mM, and the ionic Ca was 1.04 or 1.11 mM when 20 mM of EDTA or citrate was added before UF, respectively. Ca ions in the casein micelle are bound to the phosphoserine residues or are part of the CCP complexes. When EDTA or citrate was added, they compete with the phosphoserine residues and CCP in the casein micelle for the Ca ions. Moreover, solubilisation of CCP from the micelle depends on the degree of saturation of the Ca complexes formed in the solution (Holt, 1985). Therefore, Ca chelators can affect the integrity of the micellar structure to different extents (De Kort, et al., 2009).

7.3.3 EFFECT OF Ca CHELATORS ON PROTEIN CONFORMATION

The Fourier transform infrared spectroscopy (FTIR) has been used by laboratories to determine the secondary structure of biological macromolecules in milk protein analysis. The determination of protein conformation is based on the characteristic absorbance of milk proteins in the 1500 − 1700 cm^{-1} range known as the amide I and amide II bands.

Figure 7.2 shows the original FTIR spectra of skim milk with or without addition

of Ca chelators and before or after UF from 1800 cm^{-1} to 1400 cm^{-1}. These spectra are typical of all spectra that were obtained for the milk protein and consist primarily of the amide I region, 1700 cm^{-1} to 1600 cm^{-1}, and the amide II region, 1600 cm^{-1} to 1500 cm^{-1}. The major peaks for protein secondary structure included α-helix (1652 ± 2 cm^{-1}), intermolecular β−sheet (1624 – 1610 cm^{-1}), intramolecular β−sheet (1645 – 1625 cm^{-1}), β−turn (1685 – 1660 cm^{-1}), random coil (1648 – 1641 cm^{-1}), and β−antiparallel (1690 – 1700 cm^{-1}) structures and the peak located at 1658 ± 2 cm^{-1} was assigned to the large loop rather than α−helix or β−turns (Curley, et al., 1998; Farrell, et al., 2001).

The interferogram of the skim milk, as control, before and after UF (Figure 7.2A) showed that Amide I and II were clearly detected between 1690 − 1600 cm^{-1} range of wavenumber and the Amide II peak was partly overlapped with Amide I peak. The α−helix (~1650 cm^{-1}), β−sheet (~1630 and 1615 cm^{-1}), and turns (~ 1680 cm^{-1}) were detected which is in agreement with previous reports (Farrell, et al., 2001). After UF, The spectra of both Amide I and Amide II peaks did not shift, but the absorbance was higher due to increase the concentration of protein in the sample. The spectra of amide I region in milk is a function of its protein concentration (Lefevre & Subirade, 1999).

There are two overall trends observed: A) the pre-treatment of the skim milk with chelators (either EDTA or citrate) made both Amide I and Amide II peaks stronger. The intensity increased with the increase of the amount of citrate or EDTA; B) The intensity of Amide I and Amide II peaks became stronger after UF for all the samples with increase in absorbance observed for nearly all component peaks corresponding to the specific secondary structures, e.g. the α−helix (~ 1651 cm^{-1}), β−sheet extended (~ 1630 cm^{-1}) and β−sheet (~ 1614 cm^{-1}), and random coil (~ 1647 cm^{-1}) when milk was concentrated 5X. In both instances (Figure 7.2, A and B) there are no dramatic conformational changes due to the addition of chelators and the UF process as there were no change in the peak position.

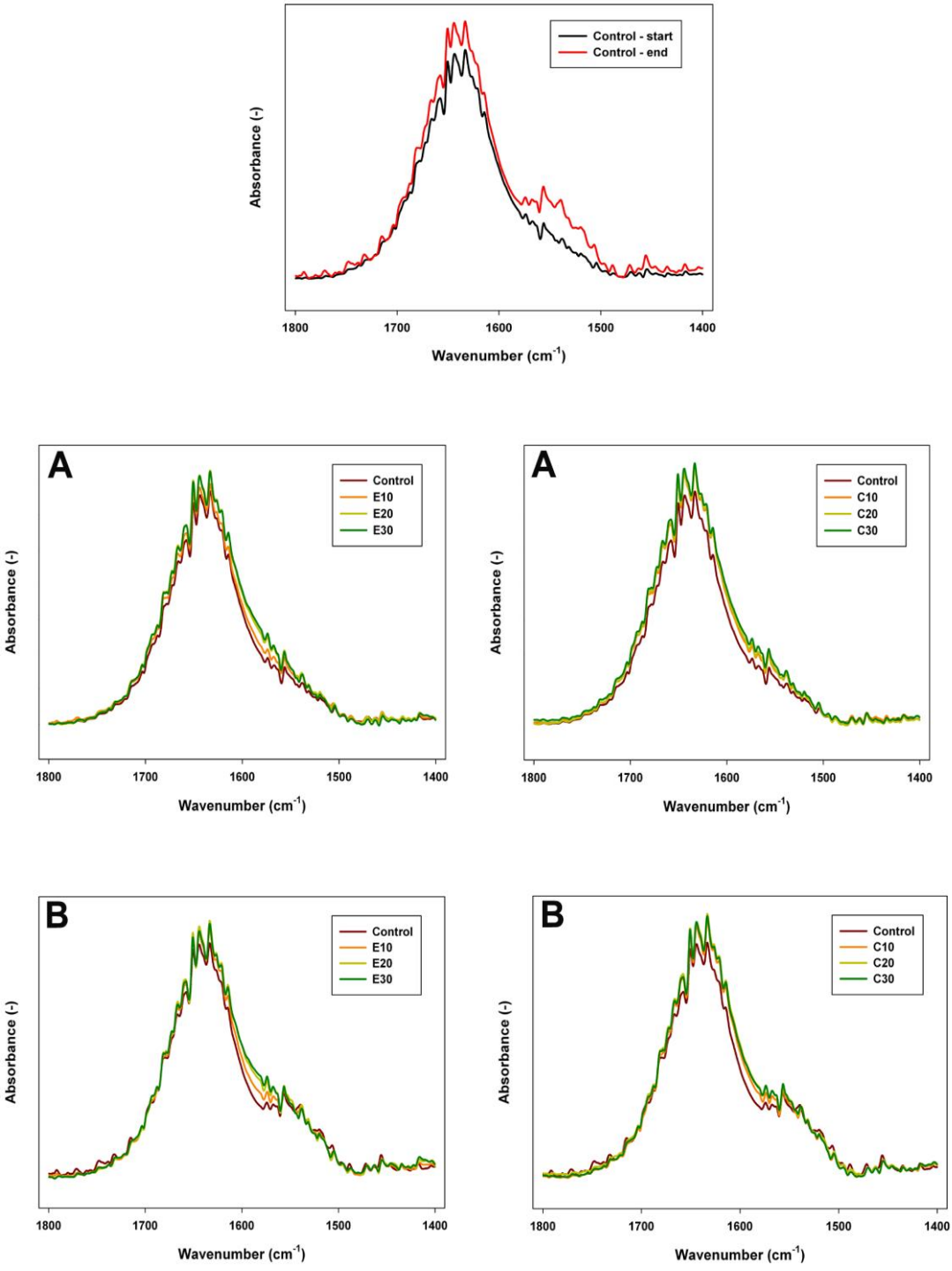


Figure 7.2. The FTIR spectrum of original skim milk and the skim milk with addition of 10, 20 or 30 mM of EDTA (left) and citrate (right) before (A), and after UF (B).

All EDTA treated samples (Figure 7.2, Left A) showed an increased absorbance in both amide I and amide II region when ≥ 10 mM of EDTA were added to the skim milk before UF. After UF (Left B), there was not much difference in amide I region when EDTA concentration increased from 10 to 30 mM, but the difference was obvious in amide II region. Figure 7.2 (Right A) presented a similar trend when skim milk was treated with 10, 20 and 30 mM of citrate. No major differences were observed in the interferograms obtained between 10 to 30 mM sample in both amide I and II regions. Only slight difference can be observed in amide II region for 30 mM of citrate treated sample, for both before and after UF. This was in agreement with the observations of Qi, et al. (2001) who found that there were little changes in the secondary structure as estimated by FTIR of native or EDTA treated proteins, particularly the κ−casein, however, the absorbance increased when citrate was added**.**

7.3.4 EFFECT OF Ca CHELATORS ON FUNCTIONALITIES OF MPC

In food applications, MPC powders are likely to be reconstituted and subjected to various treatments and processing conditions. Thus, it is important to determine the functionalities of reconstituted MPC powders to prevent process variability or losses in product quality.

7.3.4.1 Solubility

Solubility has long been a problem of commercial MPCs for the global dairy industry (Huppertz, 2010). The solubility of MPC powders have been the focus of a number of studies (Anema, et al., 2006; Fang, et al., 2011; Havea, 2006). A possible mechanism for the decrease in solubility is the formation of a monolayer of close−packed micelles at the surface of the particles (McKenna, et al., 1999) through hydrophobic and / or hydrogen bonding at the surface of the powder that could act as a barrier to water transport and subsequently inhibit the hydration of the MPC particles (Anema, et al., 2006).

In this study, the shift of ionic equilibrium between serum and micelles in milk by addition of Ca chelators and during membrane filtration and its impact on MPC solubility was determined.

The results showed that the solubility (Figure 7.3) of the MPC increased ($P < 0.05$) from 87 % (Control) to 92, 98 and 93 % when 10, 20 or 30 mM of EDTA, respectively, were added. The solubility increased ($P < 0.05$) to 94.8, 95.4 and 98 % when 10, 20 or 30 mM of citrate was added. The highest solubility was for the MPC sample prepared with 20 mM EDTA or 30 mM citrate was added. This indicated that addition of EDTA or citrate enhanced solubility depending on the amount of EDTA or citrate addition. This is in agreement with Anema, et al. (2006); Havea (2006) and Mimouni, et al. (2010), who suggested that the insolubilizing material is almost entirely made up of caseins, because the whey proteins, minerals, and lactose are more soluble. Therefore, the dissociation of casein by the addition of Ca chelating agents, as shown in this study, resulted in an increase in MPC solubility.

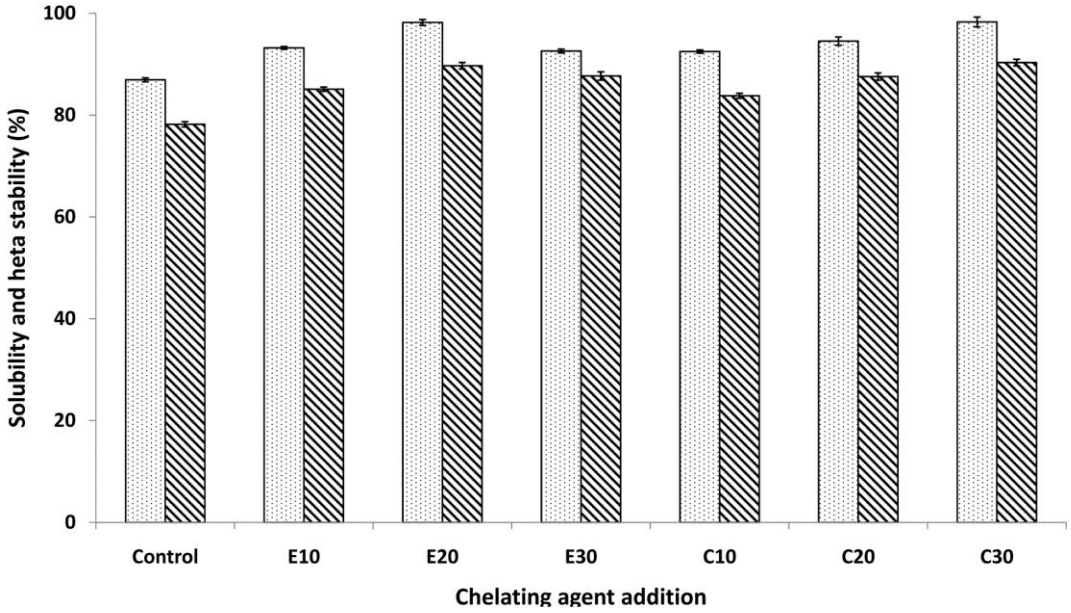


Figure 7.3. Solubility (▫) and heat stability (▨) of MPCs obtained by UF of skim milk pre-treated with 10, 20 and 30 mM of EDTA or citrate.

The solubility of MPC can be affected by addition of monovalent salts prior to evaporation or spray drying of the retentate. The improved solubility was attributed to the reduced micelle interaction and increased release of non−micellar casein (Schokker, et al., 2011), structural changes, and modifications to mineral composition (Famelart, et al., 1999) that resulted in

modified protein–protein interactions (Schuck & Demeler, 1999;). Enhanced solubility of MPC80 has also been associated with the modification of hydrophobicity and the reduction in the formation of disulphide bonds, which may reduce protein aggregation during concentration and drying. In this study, the increase of solubility could be contributed firstly to the decrease in the concentration of free Ca ions (Table 7.1) by addition of EDTA or citrate, leading to the shift in mineral equilibrium in the milk, and secondly to the shift of the ionic equilibrium between serum and micelles during membrane filtration, resulting in the dissolution of CCP and release of specific caseins from micelles, and eventually the reduction in casein micelle size of micelles (Figure 7.1). It is this reduction of micelles size that contributed to the improvement of MPC solubility (Fiure7.3). This is in agreement with the findings of Vojdani (1996) who observed that protein solubility can be improved by changing the ionic strength of the milk serum. The addition of Ca mineral to skim milk in the preparation of MPCs leading to improvement in solubility has also been reported by Schuck & Demeler (1999).

7.3.4.2 Heat stability

The lack of tertiary structures makes caseins extremely heat−stable, while whey protein denaturation is one of the main effects of milk during heating. The subsequent whey protein aggregation involves the interactions involving β−Lg, κ−casein, α−La, and BSA via –SH/S–S interchange reactions (Considine, et al., 2007), leading to irreversible aggregation of proteins into protein complexes with varying molecular size depending on the heating conditions and protein composition. Such instability manifests itself in the form of heat−induced coagulation of milk protein and has to be minimized or prevented (Fox, 1981; Heni, et al., 2014). Compositional factors influence greatly the heat−induced destabilisation of milk (Horne & Muir, 1990). The formation of complexes between β−Lg and k−casein, in the serum or colloidal phases, is associated with regions of minimum and maximum heat stability in milk (Oldfield, et al., 2000) and high Ca−ion activity (Philippe, et al., 2003; Sievanen, et al., 2008) has negative effects on heat stability.

In this study, the results of heat stability of MPC (Figure 7.3) indicated that it

increased ($P < 0.05$) from 78 % (control) when 10, 20 and 30 mM of EDTA or citrate was added. The heat stability plateaued with the addition of 20 mM EDTA and was maximum with the addition of 30 mM citrate.

The physico−chemical changes that occur in the casein micelles upon addition of Ca chelators can affect the heat stability of milk because chelators decrease the concentration of free Ca ions in the serum phase and reduce the Ca−induced protein aggregation, and, consequently, increases the heat stability of milk (Kocak & Zadow, 1985; Krieger, 1972). The results (Figure 7.3) are in agreement with these observations that Ca chelators can increase the heat stability due to a shift in the casein−mineral equilibria. This shift also increases the repulsion between negatively charged amino acid residues in the casein micelles, resulting in an increase in hydration and voluminosity of the micelles (Augustin & Clarke, 1990). However, Ca chelators may also decrease the heat stability of milk at a certain concentration, as they can chelate CCP from the casein micelle to a level at which the integrity of the micellar structure is lost (Karlsson, et al., 2005). The results from our study showed that the heat stability significantly ($P < 0.05$) increased for all the MPCs obtained from UF of skim milk treated with EDTA or citrate. However, the extent of increase was lower for MPC containing EDTA 30 as compared to EDTA 20 mM.

7.3.4.3 Emulsification properties

Emulsifying capacity (EC) and emulsion stability (ES) are two important functional characteristics of proteins that affect the behaviour of various industrial products (Wagner & Gueguen, 1999), therefore they have been used to describe the emulsifying properties of protein in food emulsion systems.

The emulsion capacity (EC)

The EC of an emulsion depends on its ability 1) to form the adsorption film around the globules and 2) to lower the interfacial tension at the oil−water interface. The results (Figure 7.4) showed an increase in EC of MPCs obtained from the skim milk added with either EDTA or citrate. The emulsion capacity increased ($P < 0.05$) from 42 (control) to 53, 55 and 60 g oil / g protein when 10, 20 and 30 mM of EDTA was added, or to 43, 50 and 52 g oil / g protein when 10, 20 and 30 mM of citrate was added. This indicated that the MPCs obtained

by UF of chelator pre-treated skim milk had enhanced emulsion capacity, the EDTA pre−treatment offered more enhancements compared to those using same levels of citrate. This is in agreement with Keowmaneechai and McClements (2002) who reported that the chelating agents (> 3.5 mM EDTA or > 5 mM citrate) reduced or prevented droplet aggregation in the whey protein-stabilized oil−in−water emulsions. The enhanced EC of the MPC by the pre-treatment of skim milk with chelating agent can also be explained by the reduced casein micelle casein micelle size. The protein with smaller casein micelle size can allow a gram of protein to cover a larger oil-water interface than a protein with larger casein micelle size (Talbot, et al., 2000). After addition of chelating agents, the casein micelle size of protein micelle was reduced (Figure 7.1), leading to the improvement of emulsion capacity.

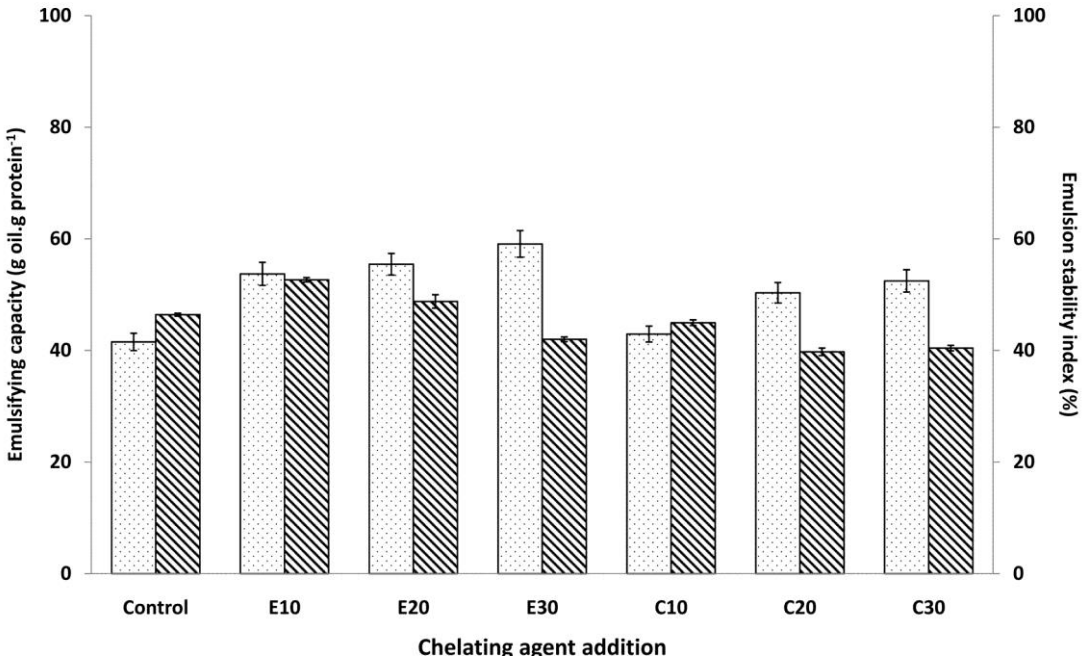


Figure 7.4. Emulsifying capacity (▫) and emulsion stability index (▨) of MPCs obtained by UF of skim milk pre-treated with 10, 20 and 30 mM of EDTA and citrate.

The emulsion stability index (ESI)

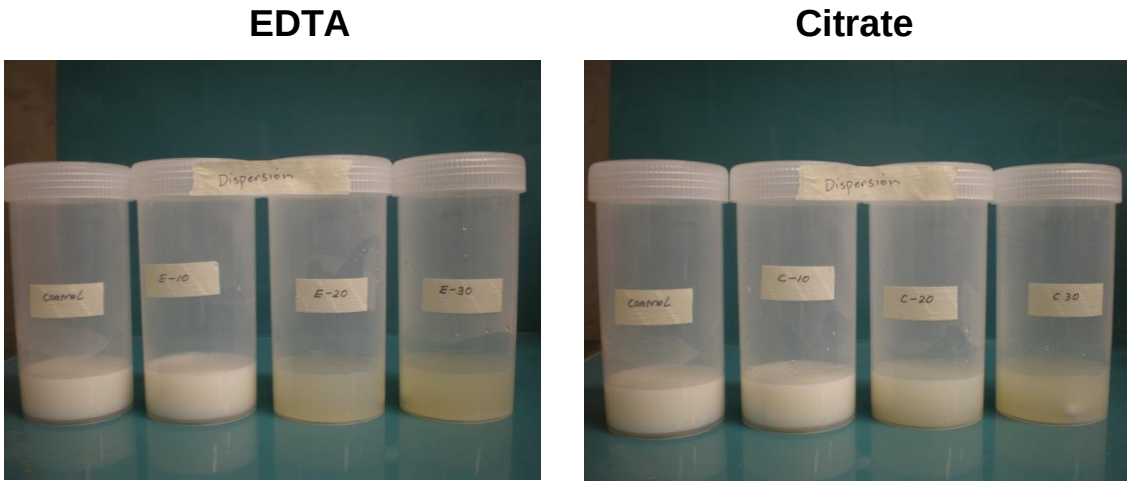
The emulsion stability is the ability of food system to resist changes in its properties over time, chemically to resist changes in chemical structure, e.g. ω−3 oxidation, citral degradation and physically, it resists changes in spatial

distribution of components over time, e.g. creaming, flocculation or coalescence. The emulsion stability is important to determine the shelf life of food emulsions.

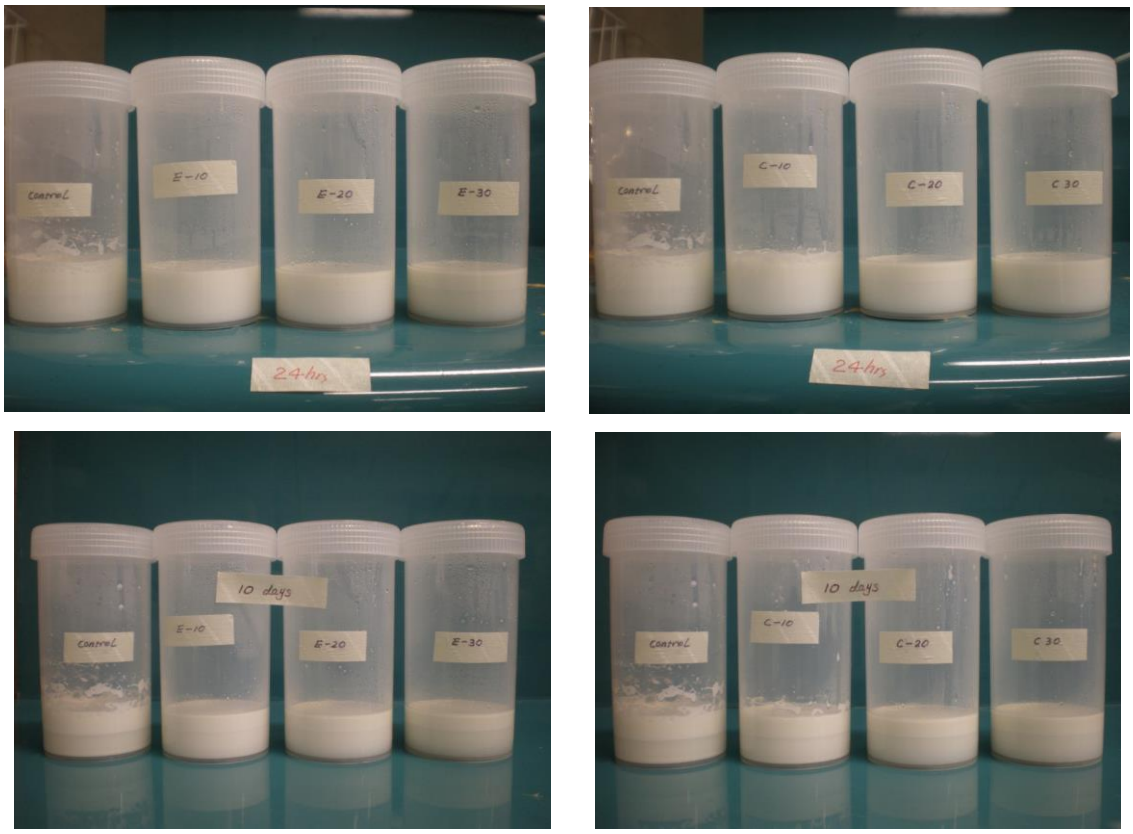
The emulsion stability of the MPCs obtained from the skim milk pre−treated with EDTA or citrate is complex (Figure 7.4). Only the MPCs from pre−treatment of 10 or 20 mM of EDTA had improved emulsion stability, being 53 % and 49 %, respectively, compared to that of control (46 %). All the other MPCs (from pre−treatment of 30 mM of EDTA or 10, 20 and 30 mM citrate) had lower emulsion stability than that of control.

Figure 7.5 shows the photographs of the dispersions and emulsions of MPCs obtained by UF of skim milk that was pre−treated with 10, 20 or 30 mM of EDTA or citrate. The dispersions of the MPCs obtained by UF of skim milk (Figure 7.5) is milky, and that pre−treated with 10 mM of EDTA and citrate are also milky, but that pre−treated with 20 or 30 mM of EDTA or citrate are less milky or transparent. The emulsion made from the MPCs obtained by UF of skim milk or skim milk pre−treated with EDTA or citrate are homogeneous at the start and after 24 h storage at 4 °C. There was an obvious creaming layer observed in all the emulsion samples after 10 days of storage at 4 °C (Figure 7.5).

The enhanced emulsion stability (Figure 7.4 and Figure 7.5) for the MPCs obtained by UF of skim milk pre−treated with 10 and 20 mM EDTA is attributed to the lower free Ca and more negatively charged droplets (Kocak & Zadow, 1985; Krieger, 1972) that improved emulsion stability. However, the fact that the emulsion stability of the MPCs obtained by UF of skim milk pre−treated with 30 mM EDTA was decreased is likely due to the loss of the integrity of micellar structure (Karlsson, et al., 2005). The reduced emulsion stability of the MPCs obtained from UF of the skim milk pre−treated with 10, 20 or 30 mM of citrate is likely due to the increased pH and the casein micelle size as found in Chapter 4. EDTA could be used at lower concentrations than citrate because of its higher Ca ion binding constant (Keowmaneechai & McClements, 2002). EDTA performed better at lower concentration than did citrate at similar level.



(A) Reconstituted MPC dispersions



(B) Emulsion

Figure 7.5. Photographs of (A) Reconstituted MPC dispersion and (B) emulsions of MPCs obtained by UF of skim milks pre-treated with 10, 20 or 30 mM of EDTA or citrate stored at 4 °C for 24 h and 10 days.

7.3.5 MEMBRANE PERFORMANCE

7.3.5.1 Flux measurement

Permeate flux is a direct measure of the membrane performance. It was hypothesised that skim milk pre−treated with Ca chelators would reduce the permeate flux due to solubilisation of hydrophobic proteins, as a result of removing CCP by chelators, and the higher the level of chelators the more effect on the permeate flux.

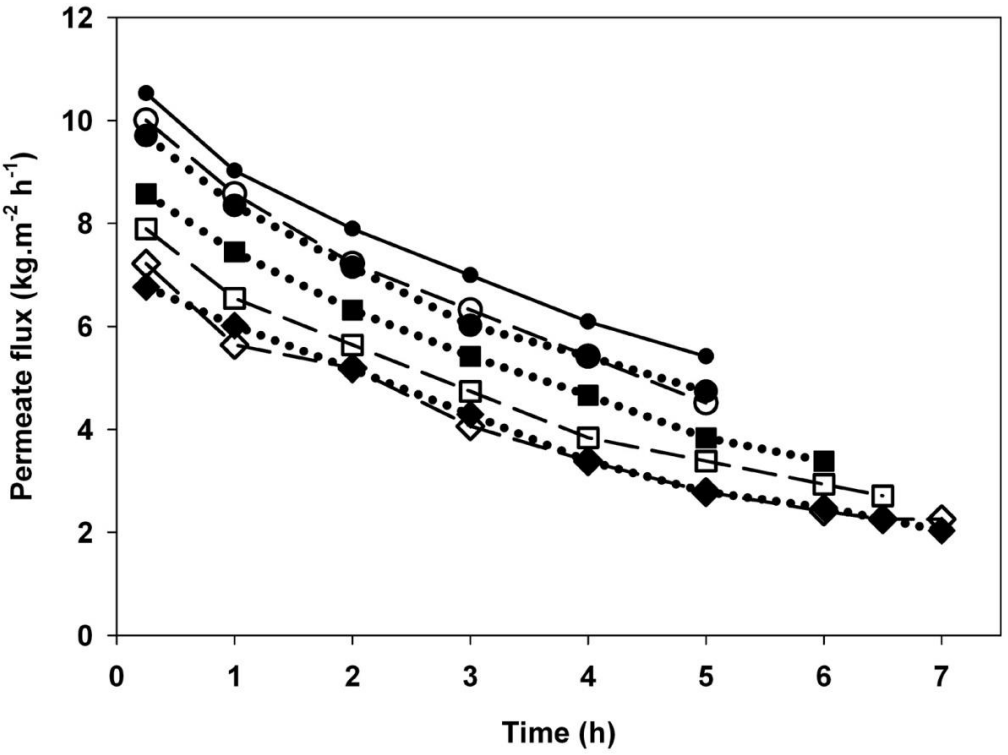


Figure 7.6. The permeate flux during UF of skim milk pre-treated with 10, 20 and 30 mM of EDTA (empty symbols/long dash lines) or citrate (filled symbols/dotted lines), —●— denotes the control. ○ denotes E10, □ denotes E20, ◊ denotes E30, ● denotes C10, ▪ denotes C20, ♦ denotes C30.

Figure 7.6 showed that the permeate flux declined steadily with time during the UF processing for the control sample as well as for the EDTA or citrate treated samples. The addition of EDTA or citrate resulted in a lower permeate flux from the start of UF process. The rank of permeate flux is control > E10 > E20 > E30 for EDTA group; and control > C10 > C20 > C30 for citrate group. The

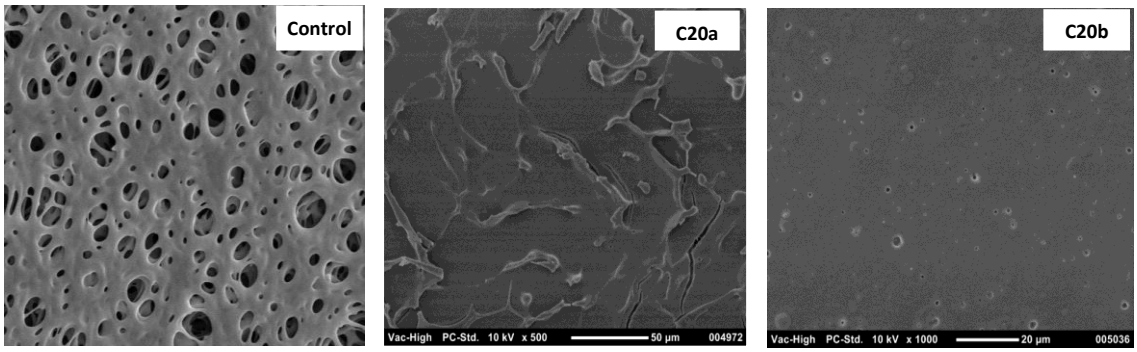
processing time increased from 5 h (control) to 6 h, 6.5 h and 7 h respectively for concentration of chelating agent at 10, 20 and 30 mM, suggesting increased fouling with increase on concentration of Ca chelator added.

7.3.5.2 SEM analysis

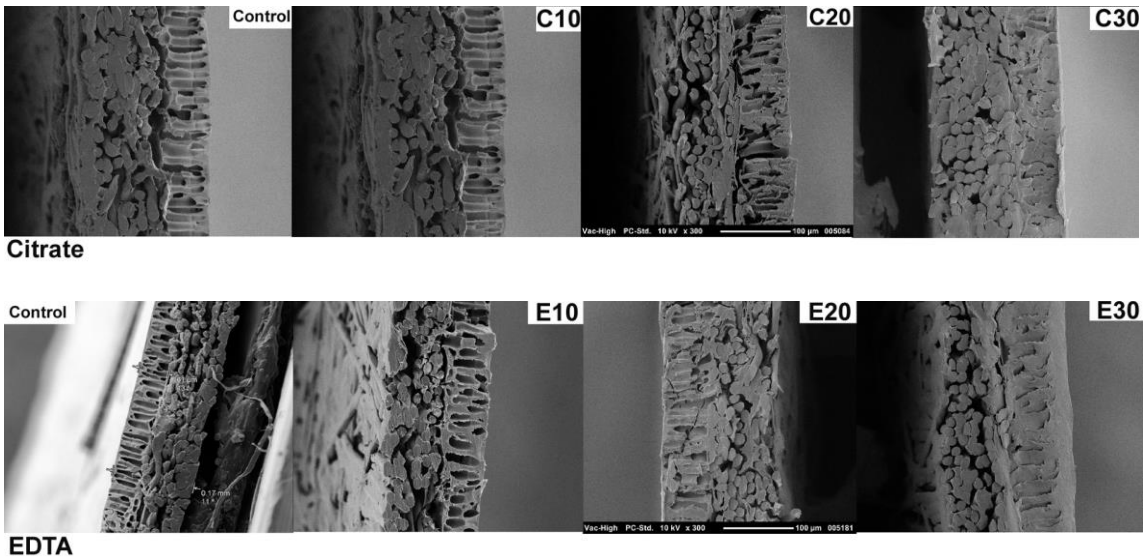
The surface and the cross sections of membranes before and after UF are shown in Figure 7.7. The surface (Figure 7.7A) of the membrane before UF appeared clean with clear porous structure (Control), and the surface of membrane used in 20 mM citrate added sample was fully covered with deposits after UF (C20 a), even after washing of the membranes(C20 b). The cross section (Figure 7.7B) of the membrane after UF of skim milk (control) was clean as observed by the presence of some clear pores on the surface and rigid cell wall in the cross section. Membrane sections obtained after UF of EDTA or citrate (10 mM) added samples presented nearly un−damaged cell wall and structure. When the chelators were added at concentration of 20 mM, damage to membranes from citrate added samples was less than that observed for EDTA added samples. A dramatic change in membrane structure was observed for the membrane after UF of skim milk pre−treated with 30 mM of EDTA and citrate. The cell wall contained a lot of small particles and the pores were heavily blocked and collated. For skim milk itself, lactose appeared to be removed from the fouling layer, whereas protein concentration generally increased, which can be explained by the fouling layer extending further across the membrane from inlet to outlet. It was suggested that casein mainly contributed in forming a strong first layer on the hydrophobic membrane surface with hydrophilic casein parts facing the feed solution, resulting in a hydrophilic coating (Hausmann, et al., 2013).

Lee and Merson (1976) reported that in UF of cottage cheese whey, membrane fouling can be caused by inorganic ions (P and Ca), which can be deposited on the membrane surface. The sequestration of Ca by chelating agents can eliminate this possibility. However removing Ca from the Ca−protein complexes can destabilize the proteins causing increase in fouling due to higher deposits of proteins on the membrane (Lee & Merson, 1976). It is known that the integrity and stability of the supramolecular structure of casein micelles are

strongly associated with their mineral content, especially CCP. When Ca−chelating agents (e.g., citrate, EDTA, polyphosphate etc.) are added to milk or micellar suspension, they induce solubilisation of CCP with consequent dissociation of casein micelles due to internal electrostatic repulsion (Lin, et al., 1972; Holt, 2004). In this study, the low concentration of EDTA or citrate (10 mM) did not cause severe fouling but critical membrane fouling occurred when 20 mM EDTA or 30 mM citrate were added. Whether Ca or released caseins or both, play a major role in membrane fouling needs to be further investigated.



(A)



(B)

Figure 7.7. The SEM images of the surface (A) and the cross sections (B) of the membranes before (Control) and after UF of skim milk pre-treated with 10, 20 and 30 mM of EDTA or citrate.

The other factor such as viscosity also could have contributed to the flux decline and membrane cell wall clogging (De Kort, et al., 2011) as increased number of small particles can induce viscosity increase. It is reported that the intact and dissociated casein micelles most likely contributed equally to the increase in viscosity, although the Ca chelators dissociated the micelles to different extents, an increase in viscosity of the same magnitude was reported by De Kort, et al. (2011). Therefore, membrane fouling increased with addition of chelators. The more EDTA and citrate added the more membrane cell wall clogged and the pores blocked.

7.4 Conclusions

Addition of Ca chelators (EDTA or citrate), at levels of 20 mM or over, resulted in reduced casein micelle size and increased zeta potential of the proteins in the skim milk. The casein micelle size was further reduced after concentration using membrane UF. This was attributed to the dissociation of mineral Ca from casein micelles, and the Ca would permeate through the membrane into the permeate stream. The EDTA had stronger effect than citrate on casein micelle size reduction, with the maximum casein micelle size reduction observed when 20 mM of EDTA or 30 mM of citrate was added. Addition of Ca chelators, either EDTA or citrate, enhanced functionalities (solubility, heat stability and emulsion activity) of the concentrated milk protein, and the functionalities reached the maximum, among the conditions tested in this study, when 20 mM of EDTA or 30 mM of citrate were added. However, there was a negative effect on the emulsion stability and membrane performance during UF. A lower flux and greater fouling took place when EDTA or citrate was added and the membrane fouling increased with the increased level of EDTA or citrate addition. Appropriate selection of a chelator and its concentration could result in MPCs with enhanced functionalities and acceptable membrane performance.

Chapter 8. **General Conclusions and Recommendations**

8.1 Introduction

MPC has become an important dairy ingredient for many applications, including processed cheese, ice cream, yogurt, fermented dairy and meal replacement beverages. Various processing conditions during milk protein concentrate production affect their physical functionalities. UF is a dairy processing unit operation used for the manufacture of cheese and dairy ingredients and in milk standardization (Gésan−Guiziou, 2013). During UF, water, soluble minerals and lactose are removed from milk system and consequently milk proteins are concentrated in the retentates (Mistry & Maubois, 2004). The effect of operation temperature, pH and addition of Ca chelating agents on the UF operation, membrane performance and the functionality of the MPC obtained was investigated in this study.

8.2 Effect of Temperature, pH and Chelators on Raw Skim Milk

Temperature and pH of raw skim milk and the addition of chelating agents affected the casein micelle size and corresponding zeta potential, soluble and ionic Ca in different ways. An increase in temperature from 15 to 50 °C led to a decrease in soluble and ionic Ca, and a decrease in zeta potential. Casein micelle size was pH dependant. At pH 6.7 and 6.3, casein micelle size was not significantly affected by the temperature, while at low pH, in particular at pH 5.5, the casein micelle size decreased slightly with the decrease in temperature. This may be attributed to the dissociation and solubilisation of some casein molecules from the micelles because of the weakening of the hydrophobic interaction between the casein molecules at lower temperature.

Adjusting the pH of the raw skim milk from 6.7 to 5.5 caused an increase in soluble and ionic Ca, and an increase in zeta potential. The effect of pH on casein micelle size was temperature dependant. At 15 °C, the casein micelle size was not significantly affected by the pH, whilst at 30 or 50 °C, the casein micelle size increased slightly when the pH was adjusted to 5.5.

Addition of EDTA or citrate caused a decrease in ionic Ca in the raw skim milk, and the addition of sufficient EDTA or citrate (≥ 20mM) caused a complete disruption of casein micelles. Addition of EDTA caused a decrease in the pH of the raw skim milk at room temperature, whereas addition of citrate caused an increase in the pH.

Overall, casein micelle size, zeta potential, soluble and ionic Ca in the skim milk are affected by and can be manipulated to a certain extent by the temperature, pH and the addition of Ca chelating agents (the type and the amount).

8.3 Effect of Temperature, pH and Chelators on UF Operation and MPC Functionalities

Temperature, pH and Ca chelators had a significant impact on the UF processing and the functionalities of the MPCs obtained.

8.3.1 TEMPERATURE

Operation temperature is one of the parameters that was found to have significant effect on the average casein micelle size of the MPC obtained. Overall, lower temperature of operation (15 °C) produced MPC with a comparably small casein micelle size, better emulsion functionality and less membrane fouling without much alteration in protein conformation, solubility and heat stability, in comparison to those produced at higher temperature (50 °C) of UF. This could be due to a greater removal of Ca into permeate, consequently leading to the greater dissociation of Ca and CCP from the casein micelles and resulting in smaller casein micelles in the final retentate and MPC. Further investigation was thus warranted to establish how additional manipulations of operating conditions, including feed modifications via pH

adjustments and addition of Ca chelators, could govern the mineral balance in milk and consequently the functionality of the MPC produced by UF.

8.3.2 pH

Pre−adjustment of the pH of the skim milk from 6.7 to 5.9 prior to UF yielded MPCs with enhanced functionalities (solubility, heat stability, emulsion activity and emulsion stability). However, further lowering of the pH of the skim milk to 5.5, some of the MPC functionalities (e.g. heat stability and emulsion stability) started to decline, and also membrane fouling has increased. To achieve the balance of obtaining MPC with enhanced functionality and membrane performance, the best pH of the skim milk prior to UF was 5.9 as assessed from this study.

8.3.3 CALCIUM CHELATORS

Addition of Ca chelators (EDTA or citrate), at levels of 20 mM or over, resulted in reduced casein micelle size and increased zeta potential of the proteins in the skim milk. The casein micelle size was further reduced after concentration using membrane UF. This was attributed to the dissociation of mineral Ca from casein micelles, and the Ca would permeate through the membrane into the permeate stream during UF process. Addition of Ca chelators, either EDTA or citrate, enhanced functionalities (solubility, heat stability and emulsion activity) of the concentrated milk protein, and the functionalities reached the maximum, among the conditions tested in this study, when 20 mM of EDTA or 30 mM of citrate were added. The EDTA had stronger effect than citrate on casein micelle size reduction, with the maximum casein micelle size reduction observed when 20 mM of EDTA or 30 mM of citrate was added. However, there was a negative effect on the emulsion stability and membrane performance during UF. A lower flux and greater fouling took place when EDTA or citrate was added and the membrane fouling increased with the increased level of EDTA or citrate addition. Appropriate selection of a chelator and its concentration could result in MPCs with enhanced functionalities and acceptable membrane performance.

8.4 General Conclusions

Careful selection of operation parameters can assist in producing MPC with a tailored functionality. Operation temperature, pH and Ca chelators are such parameters and pre-treatment options that were found to have significant effect on the average casein micelle size of the MPC obtained, resulted in changing its final application of MPC. Overall, lower temperature of operation (15 °C), moderate pH (5.9) and addition of 20mM of EDTA or 30 mM of citrate produced MPC with a comparable small casein micelle size, enhanced functionalities (solubility, heat stability, emulsion capability and emulsion stability) and reasonably good flux rate through UF membrane without much alteration in protein conformation, in comparison to those produced at higher temperature (50 °C) of UF and pH pre-adjusted and other levels of chelator added. This could be due to a greater removal of Ca in permeate and likely with some involvement of plasmin activity. Lowering pH of the skim milk from 6.7 to 5.5 prior to UF reduced the size of casein micelle in the retentate and resulted in the final MPC with enhanced solubility, heat stability, emulsion capability and emulsion stability. However, lowering the pH of the skim milk prior to UF, on the other hand, reduced the membrane performance with increased membrane fouling and took longer time to achieve similar concentration. The optimum pH was found to be 5.9 among the pH values tested in this study. Pre-treatment of skim milk feed with Ca chelators (EDTA or citrate) prior to UF reduced casein micelle size, resulted in the MPC with enhanced functionalities, but also reduced membrane performance, increased membrane fouling. The level of chelators of 20 mM of EDTA and 30 mM of citrate were selected based on the maximum functionalities tested.

This work has provided new insights into the relationship between temperature, pH and Ca chelators on the Ca content, casein micelle size in the concentrates of skim milk during and after the UF, the physicochemical properties of MPC produced, and demonstrated the potential of using temperature, pH and EDTA / citrate to manipulate UF process to produce MPC with enhanced functionalities.

8.5 Recommendations

Further study is recommended in the following areas to further scientific understanding of the membrane UF and assist industry to produce MPC with enhanced functionalities:

1. Systematic investigation of membrane fouling mechanism including using synchrotron;
2. Investigation of the functionality of the MPC, which was obtained by UF and spray drying and comparing the effect of drying method (spray drying vs freeze drying) on the MPC functionality;
3. Investigation of the moisture sorption and rehydration behaviour of the MPCs obtained from UF at different conditions and upon storage;
4. Investigation of the functionality change of the MPC during different storage conditions;
5. Investigation of scaling up of the MPC production using UF and production of prototype MPC with enhanced functionalities;
6. Optimisation of UF operation parameters (e.g. temperature) and pre-treatment conditions (pH, addition of Ca chelators), spray drying parameters, pilot scale/commercial scale production of MPC with enhanced functionalities;
7. Investigation of different types of UF membranes and their suitability for MPC production.

Chapter 9. **References**

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