Effect of Storage Conditions on the Solubility and Rennet Gelation Characteristics of Micellar Casein Concentrate (MCC).

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Abstract

Micellar casein concentrate (MCC) is a microfiltration-produced dairy low-lactose protein ingredient, consisting mainly of casein micelles in their native form with a minimal amount of whey proteins. The physicochemical characteristics and nutritional value of the protein have been utilised in a wide range of food applications such as cheese, yoghurt, ready-to-drink beverages and dairy protein-fortified products. This emerging product benefits the market growth of the dairy industry due to an increase in consumer demand towards protein-fortified foods with all-natural, minimally processed, nutritive value-added protein ingredient. So, it is vital to understand the challenges that manufacturers, supply chain and customers may face on storing the product for a period of time. The purpose of this study was to analyse the influence of storage time (180 days) and temperature (20°C, 30°C and 40°C) on the solubility and rennet gelation characteristics of MCC powder in cheese manufacturing.

The solubility of fresh and stored MCC samples was tested under various reconstitution temperatures (25, 30, 35, 40, 50 and 60°C) at 30- and 180-minute hydration times by measuring moisture content and particle size distribution using moisture-dish and Mastersizer respectively. The maximum solubility of the fresh MCC powder was found to be obtained at 50°C hydration temperature with a hydration time of 30 min, which will benefit industry by reducing the dissolving time of the powder. The solubility of stored MCC samples at various rehydration temperatures for different periods was found to decline as the storage time and storage temperature increased for any given hydration temperature. The MCC powder stored at 40°C became completely insoluble after 30 days storage. Even at the room temperature of 20°C, a linear decrease in solubility from 99% to 80% was observed over a storage period of 180 days.

The cause for the solubility decline rate in the stored MCC samples was found to be correlated with the browning index (BI) indicating that, despite the low content of lactose, Maillard reactions in the stored samples are the likely cause of insolubility. The combined microstructural analysis of scanning electron microscopy (SEM) and transmission electron microscopy (TEM) gave an insight of particle size, shape and a cross-sectional view of casein micelles in powder particles. However, protein crosslinking was not visible in the stored MCC samples.

The study found an alternative industrially available option to improve MCC solubilisation regardless of the storage conditions. By generating shear force using a single-stage high

pressure homogeniser to breakdown the particles, solubility was shown to be increased from 63% (the least soluble powder used in this section of the thesis) to 97% in the stored MCC samples.

The rheological properties of rennet gels made from skim milk-fortified fresh MCC with two different hydration times of 24 hr and 3 hr were quantified and the results were compared using two dynamic gel firmness tests, Formagraph and Low-amplitude oscillation rheometric (LAOR) analysis. The study found that 3 hr of MCC rehydration gives the optimal gelation properties which will also be benefit industry by reducing the hydration time during protein fortification. Both the test methods yielded comparable results for rennet gelation properties, but, the Formagraph had an advantage of assessing 10 samples at a time.

The influence of renneting set temperature (30ºC & 32ºC) and pH (standard sample's pH 6.7 and adjusted pH 6.3) on the rennet gelation properties of skim milk-fortified fresh MCC solution tested using only the Formagraph due to comparable results with LAOR and the test's efficiency. Increasing the renneting set temperature from 30°C to 32°C results in faster gelation time and optimal cutting time with high firmness and firming rate. Pre-acidifying the skim milk-fortified MCC samples to pH 6.3 resulted in faster gelation and cutting time with higher gel firmness and firming rate than the pH 6.7.

Due to the project's time constraints which were magnified by COVID-19 pandemic related lockdown, only five samples were selected to study the rennet gelation properties of stored MCC samples with no modifications done to the experimental set-up conditions such as temperature and pH. As the storage temperature and time increases, the solubility decreased, which resulted in longer gelation and cutting times with weaker gel formation.

This study provides novel information on the solubility and rennet gelation properties of MCC and also ingredient functionality with respect to the storage temperature and time. The research presented here has implications for product formulation in an industrial setting.

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List of Abbreviations

MCC	Micellar Casein Concentrate
MPC	Milk Protein Concentrate
LH-SMP	Low Heat-Skim Milk Powder
MF	Microfiltration
UF	Ultrafiltration
DF	Diafiltration
GMP	Glycomacropeptide
MRP's	Maillard Reaction Product's
CCP	Colloidal Calcium Phosphate
NPN	Non-Protein Nitrogen
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscope
PSD	Particle Size Distribution
HMW	High Molecular Weight
LAOR	Low-Amplitude Oscillation Rheometric Analysis
G'	Storage (or) Elastic Modulus
G"	Loss (or) Viscous Modulus
RCT	Rennet Coagulation Time
GT	Gelation Time
MGF, a30	Maximum Gel Firmness
GFR, 1/k20	Gel Firming Rate
k20	Optimal Cutting Time
SCT20Pa	Set-to-Cut Time at suitable Firmness (20Pa)
pI	Isoelectric Point
μm	Micrometre
MPa	Megapascal
H_2SO_4	Sulfuric Acid
HCl	Hydrochloric Acid
NH_4OH	Ammonium Hydroxide
$Ca(OH)_2$	Calcium Hydroxide
NaOH	Sodium Hydroxide
KOH	Potassium Hydroxide
NaCl	Sodium Chloride
$CaCl_2$	Calcium Chloride
Ca^{2+}	Calcium Ion

1. Introduction

Micellar casein concentrate (MCC) is a dairy protein ingredient that is produced by microfiltration membrane technology by retaining casein micelles in its native form. Among bovine milk proteins, caseins comprise 75-80% of the total protein are a valuable food ingredient. The physicochemical characteristics and nutritional value of the protein have been utilised in a wide range of applications such as dairy products, meat products, beverages and in baked goods (Fox & Kelly, 2004; Sauer, Doehner, & Moraru, 2012). Casein-based ingredients are produced using traditional methods such as acid precipitation, rennet coagulation or co-precipitation. As a consequence of these methods, casein micelles are disrupted and this alters the physicochemical properties of the protein (Beliciu, Sauer, & Moraru, 2012). The modern advancement of microfiltration membrane technology concentrates ~65-95% whey-reduced casein-rich product (MCC) from the skim milk without a chemical modification of the milk. Different terminologies such as “native phosphocaseinate”, “native micellar casein”, “micellar casein isolate” or “micellar casein concentrate" are used in the literature as no specific regulations are defined for MCC (Carr & Golding, 2016; Kelly et al., 2000; McSweeney & Fox, 2013).

Meena, Singh, Panjagari, and Arora (2017) described milk protein powders, which are rich in protein content are classified as “concentrate” and “isolate”. It means if the protein content in the powder less than 90% on dry matter basis is known as “milk protein concentrate” and protein content more than or equal to 90% on dry matter basis known as “milk protein isolate”. Out of two milk proteins, whey protein powders are well-established and utilised in a wide range of products. However, in contrast, micellar casein-based powders, as distinct from casein and caseinate powders, are still emerging because of their quite complex structure of casein micelles. For the application of dairy product development, micellar casein-based powders are increasingly gaining interest due to their high heat stability whereas, whey protein is quite unstable and denatures under heat treatment (Walstra & Jenness, 1984). These powders have been classified as second-generation ingredients due to their favorable functional properties such as emulsifying, gelling, foaming and nutritional properties (Kinsella & Morr, 1984; Nasser, Moreau, Jeantet, Hédoux, & Delaplace, 2017; Parkash, 1969).

Among the functional properties, powder rehydration ability is the most important and primary factor to be considered. In view of the fact that to evolve its full functional properties for most

applications, MCC powder should be completely dissolved in water. Subsequently, solubility and rehydration are the primary pre-requisite properties of MCC powder and needs exact evaluation (Bouvier, Collado, Gardiner, Scott, & Schuck, 2013; Gaiani et al., 2006; Nasser et al., 2017). In particular, Richard et al. (2013) performed experimental investigations on the rehydration conditions of the micellar casein-enriched powders and using a total rehydration time of more than 180 min at room temperature (25°C) under stirring speed of 900 rpm. However, some undissolved material sediments were found to remain and are known as "insoluble fraction". Therefore, the present study intends to optimise rehydration time with a high solubility rate for the benefit of economic industrial applications.

The use of MCC to fortify milk for cheese making or to substitute milk altogether is very sensible because the protein in cheese is mostly casein. When estimated using a nonlinear programming optimization method, (Papadatos, Neocleous, Berger, & Barbano, 2003) showed an increase in net profit for the cheesemaker when microfiltration (MF) was done prior to cheese-making. The disadvantage of a common alternative protein fortifier, milk protein concentrate (MPC), is that, the whey protein fraction was lost during the cheese production and does not contribute to cheese yield (Carr & Golding, 2016). As the whey protein composition in MCC is very low, it can be utilised as a cheese milk extender to overcome the issue. So, as a product manufacturer, it is significant to understand each functional property with its application oriented.

In the perspective of exporting the MCC powder to different countries distribution channels and shipping can result in the powder being stored at high temperatures and for extended storage times. Many researchers have proved solubility decreases at a higher rate at high temperatures in high-protein (MPC) content powder (Nasser et al., 2018; Anema et al., 2006). Among the various available forms of MCC, powder makes it easy for industrialists to export and store it for quite a long time. In this research, a commercial powder was used to understand the functional properties with the powders stored at three different temperatures (20°, 30° & 40°C).

Based on consumer demands and expectations towards high-protein-based products, MCC stands-out to be unique in comparison to traditional casein ingredients with broad applications in the food and dairy industry. The functional properties of MCC make it a useful choice for the applications of high-protein-low-lactose beverages, high-protein nutritional bars, cheese-making and yogurt production. However, a few works of literature state MCC's application is

restricted because of its poor rehydration characteristics associated with the higher protein content. While there is much literature available on the production and concentration of MCC, there is considerably less information on the understanding of functional properties oriented to final application. Thus, the literature review structures the casein protein characteristics with commercially available casein ingredients; the unique characteristics of MCC in comparison to other casein products; the principle components, the manufacturing process, the physicochemical properties and the applications of MCC.

2. Literature Review

2.1 Casein Protein Characteristics

Milk protein provides remarkable nutritional benefits and their high level of functionality keeps them popular in the human diet. From milk, ease of protein isolation process leads to the production of various nutritional milk protein ingredients yet additionally these are distinguished among other food proteins. The major portion of milk protein in bovine milk is comprised of casein (~75-80%), a phosphoprotein that can be precipitated from milk at pH 4.6 at 20°C through acidification process (McSweeney & Fox, 2013). Casein is further classified into four well-known subgroups (α_{S1}-, α_{S2}-, β and κ-caseins), and their general properties are illustrated in Table 2.1. The distinctive nutritional aspect of casein provides essential amino acids required in the human body with the addition of calcium content, which is essential for bone health.

Table 2.1 Casein Molecules - General Properties (Broyard & Gaucheron, 2015).

	α_{s1}-casein (B variant)	α_{s2}-casein (A variant)	β-casein (A^2 variant)	κ-casein (B variant)
Molecular weight (Da)	23 614	25 230	23 983	19 023
Concentration ($g.L^{-1}$)	10	2.6	9.3	3.3
Amino acids	199	207	209	169
Proline	17	10	35	20
Cysteine	0	2	0	2
Glutamic acid	24	25	18	13
Aspartic acid	8	11	5	1
Phosphoserine	8	11	5	1
Apolar residues (%)	36	40	33	33
Glycosylation	0	0	0	0–5
Charge at pH 6.6	−21	−15	−12	−3

Furthermore, the high contents of prolyl residues form open and flexible conformations. Thus, casein molecules are excellent in surface-active and stabilizing properties (Broyard & Gaucheron, 2015). Some commercially available casein-based protein products are caseinates, acid caseins, rennet caseins and milk protein concentrates. Modern innovations in membrane filtration of microfiltration have enabled separation of casein micelles from whey to manufacture a new native casein concentrates product known as MCC. It is noteworthy to understand the MCC difference from commercially available casein products.

2.2 Commercially Available Casein Ingredients

2.2.1. Acid casein

Acid caseins are obtained by isoelectric precipitation of casein that can be achieved by the addition of mineral acids (such as 1.0 N H_2SO_4 or HCl) or either by bacterial fermentation process where lactose is converted into lactic acid bacteria. The acidification process decreases the pH to the isoelectric point of casein (pI 4.6) and dissolves the colloidal calcium phosphate hence results in low mineral content. It is an insoluble product in water and further processing steps are required to yield soluble ingredients.

In contrast, MCC retains bound minerals associated with the micellar structure. The membrane filtration of skim milk yields a direct purified form of colloidally stable casein in their native form minimising the need for the additional complex production process of acid casein (Carr & Golding, 2016).

2.2.2 Rennet Casein

Rennet caseins are produced by the addition of proteolytic enzyme (rennet) to the skim milk which results in the cleavage of the peptide bond in κ-casein known as glycomacropeptide (GMP) depicted in Figure 2.1. This protein destabilisation process reduces the zeta potential of the casein micelles and thus, reduces electrostatic repulsion and results in curd formation (protein aggregation). Unlike acid casein, the coagulation occurs at the pH of the milk and therefore casein-associated minerals such as calcium phosphate are retained. The solubility of rennet casein is quite low; however, it can be solubilised by increasing pH or by the addition of calcium sequestering agents (Mulvihill & Ennis, 2003).

$$\kappa \rightarrow \text{casein} \xrightarrow{\text{chymosin}} \text{para} - \text{k} - \text{casein} + \textit{glycomacropeptide}$$

$$\text{para - k - casein} \xrightarrow{Ca^{2+}} \textit{Dicalcium}\ \text{para - k - casein}$$

Figure 2.1 Two-Stage Process of Rennet Casein Production (Carr & Golding, 2016).

In contrast to rennet casein during MCC manufacture, loss of GMP can be avoided. Also, with MCC these additives, required to solubilise rennet casein, can be avoided enabling increased sales based on growing trends for “clean-label” or “E-number free products” (Carr & Golding, 2016).

2.2.3 Caseinates

Caseinates are produced by neutralizing acid casein by the addition of alkali such as NH_4OH, $Ca(OH)_2$, NaOH and KOH. By dissolving acid casein in an increased pH environment, it tends to be water-soluble. The final intramolecular caseinate structure and functional properties can be manipulated and allow the manufacturer to produce customised ingredients. The caseinates solution is viscous in nature with 20% limited solids for ease of handling during the manufacturing process. Consequently, the drying process efficiency is low. One of the important tools for customisation of caseinates is by regulating the mineral balance and this worked as the basis for the alteration of membrane produced protein, milk protein concentrates (MPC) (Carr & Golding, 2016; Fox & McSweeney, 1998).

2.2.4 Milk Protein Concentrates (MPC)

Among casein-based products, this is the first membrane produced product on the market. MPC is manufactured by concentrating the skim milk by ultrafiltration, which retains casein micelles and whey protein and permeates water, lactose, soluble salts and non-protein nitrogen compounds. However, minerals such as calcium phosphate, magnesium and citrate with the casein micelle structure are concentrated and present in MPC. One target market of MPC is its use as a cheese milk extender. In the past, the solubility characteristics of MPC remained the biggest barrier to sales. This was overcome by finding the solution through mineral balance alteration (Carr, Bhaskar, & Ram, 2004), and calcium concentration reduction (Bhaskar, Singh, & Blazey, 2001). However, the whey protein fraction of MPC is lost during the cheese production and did not contribute to cheese yield (Carr & Golding, 2016).

The major difference between MPC and MCC is that MCC has a much lower level of whey proteins and therefore would contribute less whey loss in cheese milk extension applications.

2.2.5 Micellar Casein Concentrate (MCC)

The novel chemical-free, high-protein content dairy product, micellar casein concentrate has been available in the industry from the 1990s, but has received less focus than MPC. The distinctive characteristics of this product (Pierre, Fauquant, Le Graet, Piot, & Maubois, 1992; Saboyainsta & Maubois, 2000), in comparison to other dairy proteins, are:-

1. Native casein micelle structure is retained in the product as its original form in the milk.

2. As the name suggests, concentrated casein micelle is present predominately with less than 5% of whey protein present.

The main focus of the published research has been with the hunt for an efficient manufacturing process. The outline sketch of product composition, manufacturing techniques, applications and functional characteristics are illustrated in Figure 2.2 and a detailed review of each attribute will be followed.

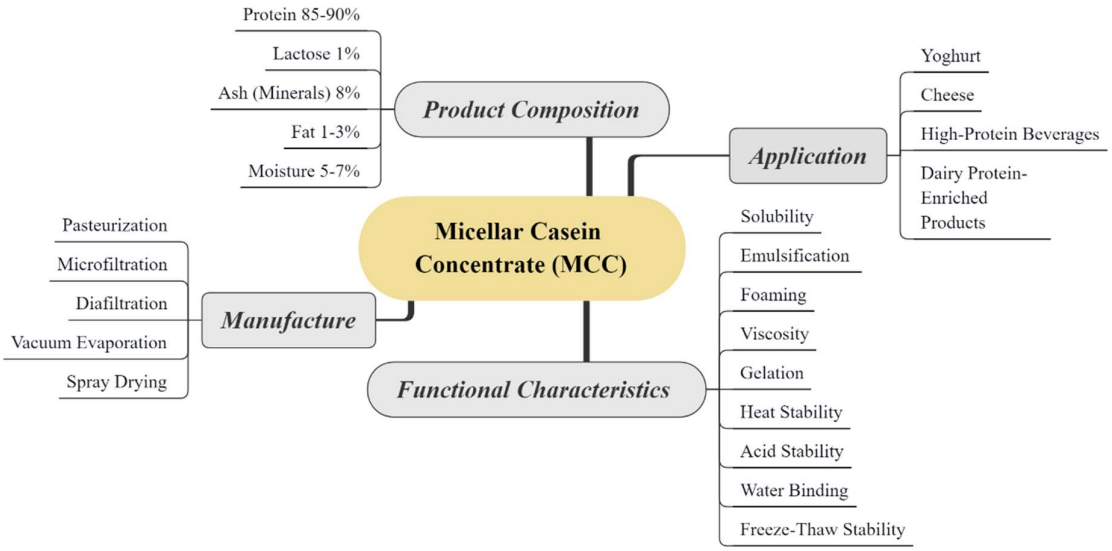


Figure 2.2 Schematic Representation of Micellar Casein Concentrates (MCC).

2.3 MCC Product Composition

2.3.1 Casein Micelles

The four sub-group casein molecules self-associate resulting in large cluster formation known as casein micelles. Out of four molecules, the α_S- and β-casein are concentrated in the middle and κ-casein on the micelle surface and thus, considered to be roughly spherical (see Figure 2.3) with the average diameter of 150 nm (Bylund, 2015). The most significant characteristics of micelles are the glycosylated form of κ-casein, which helps it to be hydrophilic and negatively charged in its C-terminal part. This is one of the important attributes for micellar structure and steric stability (Broyard & Gaucheron, 2015). These micelles are linked together by "nanoclusters" of calcium phosphate and hydrophobic forces between protein groups holding the micelles together (Thompson, Boland, & Singh, 2009). However, the internal structure of casein micelle is proposed by three main models namely; the nanocluster, the dual-binding and the sub-micelle model because of the dispute about the exact structure.

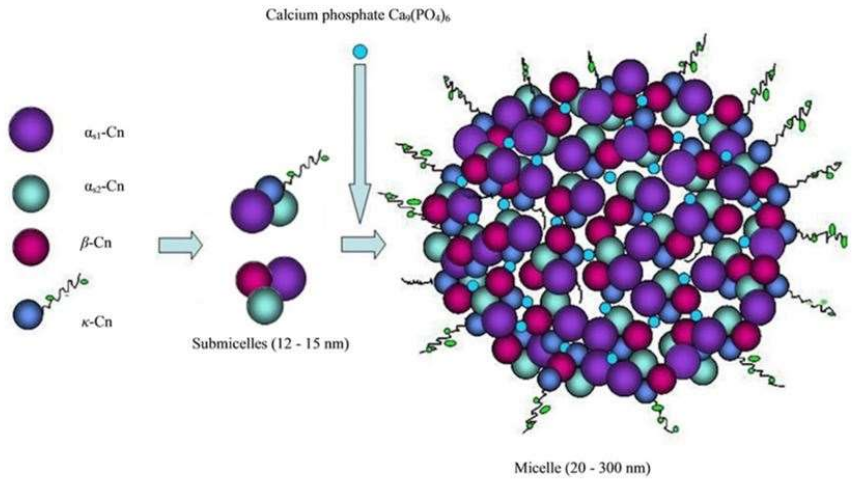


Figure 2.3 Structure of casein micelles composed of submicelles and linked together by calcium phosphate (Rebouillat & Ortega-Requena, 2015).

Several authors stated that certain criteria should be met for the valid micelle model. The micelle surface surrounded by a κ-casein layer, its location should be able to stabilize α_S- and β-casein proteins. Then, the serum proteins (β-lactoglobulin) and κ-casein will be able to form complexes in the presence of high temperatures. The enzyme such as chymosin, a bulky protease will be able to hydrolyse the outer layer, κ-casein. The casein micelle structure reacts to micellar environment change, pH, temperature and pressure and thus it is considered to be a flexible dynamic structure (Damodaran & Parkin, 2017). When acidification takes place and, the natural pH of the milk decreases to the pI of the casein micelles, it leads to the loss of steric repulsion between micelles and there will be a breakdown in the polymer brush which contributes to an aggregation of the colloidal network. Lowering the pH also causes loss of calcium phosphate as it solubilises and migrates into the serum phase (de Kruif & Zhulina, 1996).

2.3.2 Lactose

In milk, lactose is the principal carbohydrate and is a reducing disaccharide. The hemiacetal lactose structure classified as α or β in an interchanged mutarotation form plays a crucial role in its physical and chemical properties. During rapid drying, the presence of lactose in solution results in an amorphous state, which is hygroscopic, and thus, it increases the rehydration at powder particle surfaces (Fox & McSweeney, 1998; Walstra & Jenness, 1984). In MCC, lactose represents a minor content of 1 to 3% the same as in MPC. Anema, Pinder, Hunter, and Hemar (2006) in their research related to MPC stated loss of solubility might be correlated with

aggregation and Maillard reaction products (MRPs). The chemical reaction of the reducing sugar (lactose) and amino (lysine) group of protein residues results in MRPs and it is known as a protein lactosylation reaction. Similarly, Gazi and Huppertz (2015) reported that theoretically presence of lactose content may possibly be the reason for the initiation of protein-crosslinking. Certainly, the presence of the minor lactose quantity cannot be neglected when we are in search of a reason for solubility loss concerning the product's shelf-life.

2.3.3 Minerals

According to Fox and McSweeney (1998), the majority of mineral salts in the bovine milk are anions of citrates, chlorides, phosphates, sulphates, carbonates and bicarbonates associated with the main cations of sodium, potassium, calcium and magnesium. These elements play an important role as nutrients and, it impacts milk protein (primarily casein's) structural conformation and stability during manufacturing and storage of milk products. Out of all the mineral salts, calcium and phosphate are particularly important due to their high concentration and further, that they are partly soluble with the insoluble fraction in a colloidal form of within the casein micelle. These two mineral salts form a covalent bond with casein micelle which is referred to as colloidal calcium phosphate (CCP), where it neutralises the protein's phosphoseryl residues and acts as bridging factor between casein molecules. This phenomenon is represented in Figure 2.4 (Schmidt, 1982).

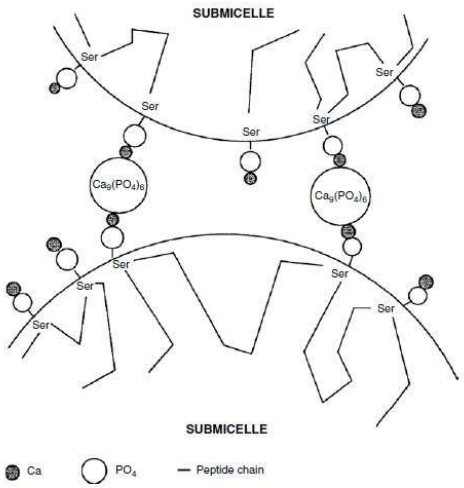


Figure 2.4 Colloidal Calcium Phosphate-Casein Micelle Complexes (Schmidt, 1982).

Due to the importance of the casein micelle in MCC and its dynamic relationship with the environment, it is necessary to understand the equilibrium effect of changes in ionic strength due to temperature and pH. Calcium salts are soluble at low pH and generally have retrograde

solubility and thus their solubility of will be higher at low temperature (Dalgleish & Corredig, 2012; Holt, Carver, Ecroyd, & Thorn, 2013).

2.4 Manufacturing Process of MCC

MCC can be produced in different protein concentrations of 50-90% (dry basis) using the microfiltration process with additional diafiltration for further purification of high protein products. Then, evaporation and spray-drying to remove water and produce a powder. Figure 2.5 shows a general manufacture process flow diagram for MCC. From this technology, the retentate generates native casein micelle structure-retained high protein MCC powder and, permeate can be utilised to produce whey products such as whey protein concentrate or isolate.

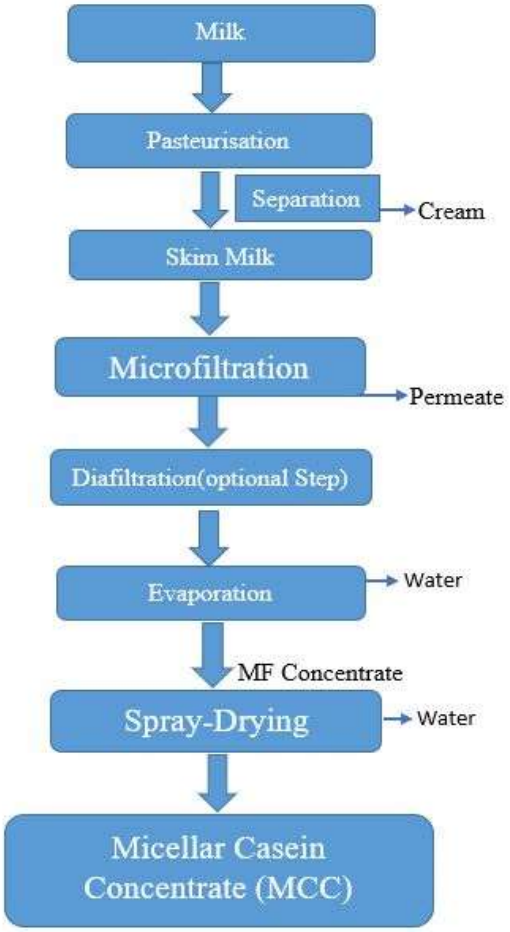


Figure 2.5 Flow Diagram of MCC Manufacturing Process.

2.4.1 Microfiltration

Traditionally, microfiltration (MF) has been specially designed for bacterial removal resulting in the production of extended shelf-life milk and also for the pre-treatment of cheese milk. It is

a cross-flow membrane filtration method, also referred to as tangential filtration in which feed (liquid) flows tangentially towards the membrane with added-advantage to overcome fouling (Bylund, 2015; Walstra & Jenness, 1984). It has the largest pore size in the range of 0.1-10 μm with the lowest processing pressure of 0.01-0.2 MPa.

Recently, MF, a pressure-driven separation method, has been adopted for the production of casein in its native form using semi-permeable membranes with a pore size of 0.1 μm (Pierre et al., 1992). Broyard and Gaucheron (2015) described MF as very useful technique in milk protein fractionation and the separation of components are based on their particle sizes. MCC can be obtained from full-fat milk or skimmed milk and casein can be concentrated to account for 95% of the total protein. The microfiltration method along with a diafiltration (DF) stage leads to the production of this form of high-protein casein retentate (Rosenberg, 1995).

There is a large volume of published studies describing the membranes commonly used in dairy processing for MF. There are two sizes typically used: - 0.1 μm for casein and whey protein separation, 1.4 μm for bacterial and fat removal. When a 0.1 μm pore size membrane used, native micellar casein is retained in the retentate because approximately average casein micelle size varies in the range of 50-500 nm (Swaisgood, 2003). Whereas in contrast, native whey protein (3-6 nm), smaller in size compared to micellar casein protein and the membrane pore size will pass through the membrane. Mostly, a small amount of whey proteins is retained with micellar casein after filtration.

In a review conducted by Maubois (2002), it was shown that casein concentration has historically been achieved with ceramic microfiltration membranes using the concept of uniform transmembrane pressure (UTP) with high tangential flow rate (>6 m/s) and filtration temperature of 50-55°C. Many researchers found the usage of the MF membranes with larger pore sizes cause membrane fouling. Brans, Schroën, Van der Sman, and Boom (2004) conducted a significant analysis and presented a detailed report to overcome fouling by maintaining unique pore size distribution with high crossflow velocity in ceramic membranes. However, this approach is not feasible for spiral wound membranes as they can be damaged with a high flow rate. Also, uniform transmembrane pressure (UTP) theory was developed for microfiltration technique to maintain low-pressure variation between inlet and outlet.

From 1990, several attempts have been conducted by the researchers to find the best-operating conditions for the maximum removal of serum protein by avoiding fouling. Initially, authors have tested protein recovery using two major membranes types; i) Ceramic (UTP, Gradient

Permeability (GP) and Isoflux) ii) Spiral-wound (PVDF polymer-based membrane). A study by Zulewska, Newbold, and Barbano (2009) compared the efficiency of serum protein removal using three membranes, bleed-and-feed 3-stage process at 50°C and reported 64.40% for UTP followed by 61.04% for GP and finally, 38.62% for SW microfiltration membranes. In a follow-up study, Beckman, Zulewska, Newbold, and Barbano (2010) found that under the same conditions, serum protein removal rate can be improved to 95% for UTP and 70.3% for SW by accompanying 2-DF stage with bleed-and feed 3-stage MF process.

The modern advancement in microfiltration leads to the extraction of native micellar casein containing CCP, however, it is noteworthy to understand the protein's physicochemical properties are altered although the resulting retentate closely resemble the native micellar casein complex. A recent study by Crowley et al. (2018) studied the impact of cold (<10°C) and warm (50°C) temperature of MF/DF on the solubility characteristics of MCC powders and found that the cold MF/DF improves the powder rehydration with an additional benefits of reduced membrane fouling and better microbial control. But, rennet gelation properties are strongly influenced by the processing methods and serum composition (Corredig, Nair, Li, Eshpari, & Zhao, 2019). In the follow-up study, the authors found that the usage of diafiltration significantly affect the aggregation behaviour of rennet-induced casein micelle suspensions due to the change in ionic equilibrium between the colloidal and soluble phase of calcium (Zhao, Renhe, Fu, & Corredig, 2020). Regardless of these physicochemical changes, the technique has got commercial value as it offers the possibility of achieving both the fractionation and concentration of ingredients in liquid systems and still the resulting components have desirable physico-chemical characteristics.

2.4.2 Spray-drying

Spray drying is the most commonly used technique in the dairy industry for powder production. Spray drying provides the advantages of easy handling, reduces transport cost by decreasing water weight, and has got longer shelf-life during storage without the need for refrigeration (Schuck, 2002). Before spray drying, the initial feed undergoes vacuum assisted evaporation to reduce the energy cost of drying from a low solid's material. Then, the concentrated feed is transferred to the drying tower. The spray drying process is explained in the following Figure 2.6. Bylund (2015) explains drying tower process can be divided into three phases; 1) Atomization 2) Evaporation 3) Collection of the dried final product.

Figure 2.6 Spray Drying Process (Sosnik & Seremeta, 2015).

Spray drying process takes place using either a spinning disk atomiser or a series of high-pressure nozzles in a large drying chamber of hot air flow temperature up to 200°C. Then, the evaporation cools down the milk droplets and helps the milk droplets not to reach the air temperature (Pearce, 2017). A cyclone is a device for the separation of air and finely powdered particles. The spray dryer operation and design can be used to control powder particles and drying efficiency. McSweeney and Fox (2013) reported two or three-stage spray drying provides better control of powder properties and greater process efficiency by balancing the rate of heat introduction with the evaporation rate.

However, Singh (2009) states that during initial feed concentration, several physicochemical properties of the feed are changed. Among several, the most noticeable changes are in the rheological nature of the fluid: as the viscosity increases, the concentrate changes from Newtonian into the shear-thinning fluid. Due to concentration during evaporation the system is becomes tightly packed and the caseins aggregated by hydrophobic bonding. Additionally, protein conformation and salt equilibrium changed. The application of heat creates a shift in the mineral equilibrium of the colloidal phase. As a result, solubility of the concentrations of soluble calcium and phosphate in reconstituted skim milk decreases by about 20% in comparison to the original milk.

Changes in protein structure are also possible. As mentioned earlier increased concentration, during evaporation, can lead to increased protein association and thus large aggregates. Sadek et al. (2013) noted that if the temperature of the concentrate in the evaporator is in the range of 65-75°C, then serum protein denaturation occurs and this can lead to increase the size of casein micelles. These authors concluded that such whey denaturation occurs only during concentration in comparison to the remaining spray drying process.

2.5 Applications of MCC

2.5.1 High-Protein Beverages

Increasingly, consumers are selective in choosing beverage and food products with low sugar and high protein content. This expectation makes MCC a suitable dairy ingredient for the application of high-protein, low-carbohydrate drink applications such as sports drinks and meal replacement drinks. The high-temperature stability property of MCC adds an advantage for beverage applications. MCC has a very high heat stable property till the sterilization temperature of 141°C, however there are mineral equilibrium changes and micelle disintegration occurs (Sauer & Moraru, 2012). The author suggested this aggregation can be avoided by increasing the pH or lowering the heat treatment temperature, or both. Also, in the absence of fat, MCC is very bland in taste and can have improved mouthfeel (Jørgensen et al., 2019).

2.5.2 Yoghurt

Mellentin (2013) claims the consumer demand for high-protein yoghurt has been predicted to increase in recent years. Specifically, protein interests are also attributed to the weight loss movement and consumers interest in 'clean-label' limited-additive goods. Recently, Jørgensen et al. (2019) reviewed high-protein yoghurt processing and noted that post fermentation acidification resulting in whey syneresis after yoghurt production, remains as key issue in the dairy sector. To overcome this issue, the review concluded that the solution offered by Bong and Moraru (2014) of protein fortification using MCC offered the most promise. The author concluded MCC-58 would be the best source for protein fortification which enables the physical properties of Greek-style yoghurt without the traditional acid whey-removal process.

2.5.3 Cheese

In cheese, the major protein constituent is casein and therefore it is very sensible to fortify milk with MCC for cheese making or to substitute milk altogether. Papadatos et al. (2003) evaluated the impact of microfiltration and found that the net revenue of cheese making is increased when microfiltered milk is used to produce cheese. The economic advantages of using MF before cheesemaking include improving plant efficiency and enabling the production of valuable co-products from the MF permeate. These results were similar to Caron, St-Gelais, and Pouliot (1997) study in which, ultrafiltered milk retentate and diafiltered microfiltered milk retentate powders were compared for the protein enrichment of milk coagulation. The increase in gel firmness with high protein-fortified milk were observed from diafiltered microfiltered milk

retentate powder in comparison to ultrafiltered milk retentate. The possible reason might be due to higher levels of calcium complexed with casein and retardation of rennet diffusion in higher protein cheese milk. The detailed comparison of UF and MF-produced cheese is discussed in the Section 2.8.2.

As the fat-content of MCC is very minimal, many authors have worked on the production of low-fat cheddar cheese. Nelson and Barbano (2004) developed an innovative method to produce 50% fat reduced cheddar cheese and succeeded by retaining the strong flavour as the original full-fat cheddar cheese and with improved soft texture than the full-fat cheese. Similar to this approach in a continuation of the study, this group later made to develop 82% fat reduced cheddar cheese (Amelia, Drake, Nelson, & Barbano, 2013). However, the flavour of low-fat cheese resulted in bitter and off-flavour and the authors recommended improvement by the addition of a flavouring ingredient. Most, studies have only been carried out on fresh microfiltration milk and very few studies have been done on reconstituted MCC powder formulation.

2.6 Functional Properties of MCC

Several authors proposed that the casein micelle can be thought of as a "functional aggregate" because of its tendency to form a fibril, planar and polygonal aggregates (Glantz, Hakansson, Lindmark Månsson, Paulsson, & Nilsson, 2010; Holt et al., 2013). This propensity of casein micelles might be a contributing factor for the high film-forming capacity of MCC. Hurt, Zulewska, Newbold, and Barbano (2010) reports during the MCC manufacturing process, ~70 to 90% of serum protein along with lactose and minerals are removed, which results in certain modification on the functional properties of MCC. Subsequently, Sauer et al. (2012) studied the effect of serum protein removal on viscosity and concluded that casein as the principle contributor to MCC viscosity as it increases with respect to high serum protein removal. Kaliappan and Lucey (2011) postulated and observed the increase in whiteness as the casein concentration increases. Most of the studies admitted the reason for the whiteness of the milk is mainly due to casein micelle's light diffraction. Protein functional properties are those characteristics that define its effective use in food products. Among the functional properties illustrated in Figure 2.2, solubility and rennet gelation characteristics will be reviewed in detail owing to the focus of the study.

2.7 Solubility

Solubility is considered as a primary and key determinant of the protein quality, which also influences the other functional properties such as gelling, foaming, thickening and emulsifying. The insolubility nature of protein results in limited food applications. Protein solubility is determined by the interaction of protein's surface hydrophobicity (protein-protein) and hydrophilicity (protein-water) with the surrounding water. The solubility would be affected by the nonpolar groups on the outside because hydrophobic residues are embedded inside the protein. If the hydrophobic surface area relative to total surface area is low, then the solubility rate would be higher. In other words, hydrophobic and attractive van der Waals interaction favours precipitation and repulsive electrostatic interaction favours solubilisation. Proteins aggregation results in protein insolubility in water and it is due to the balance of interaction force among the molecules (Damodaran & Parkin, 2017).

$$Protein - Protein + Water \rightleftarrows Protein - Water$$

Figure 2.7 Force involved in protein solubility (Damodaran & Parkin, 2017).

Rehydration is a dynamic process with a mixture of phases such as wetting, swelling, sinking, dispersing and dissolution and these stages overlap or occur concurrently (Crowley, Kelly, Schuck, Jeantet, & O'Mahony, 2016). The rehydration activity is influenced by various factors such as raw material composition, processing methods, reconstitution conditions such as stirring speed and temperature, the resulting structural variation such as the presence of pores and agglomeration (Kinsella & Morr, 1984; Parkash, 1969). The rehydration issues vary with different dairy powders for instance; wetting is the limiting step for whey protein powders while dispersion is the limiting step for casein-based powders. Based on the limiting step, product quality may be affected by the lack of protein expression or the formation of lumps with non-hydrated regions (King, 1965).

Several authors investigated the dispersion and dissolution step as it is the limiting step for casein-based powders. Mimouni, Deeth, Whittaker, Gidley, and Bhandari (2010b) proposed that the breakdown of agglomerates into the primary particles for high protein-based powders involves two classes of dissolving components; 1) Slow-dissolving components such as colloidal calcium phosphate (CCP) and casein 2) Fast-dissolving components such as lactose, minerals and serum proteins. These were in agreement between the King (1965) and Parkash (1969) studies and the authors further explained that the slow dissolving components for casein-based powders are mainly due to the presence of a surface skin layer of interlinked casein micelles present on the particle surface preventing effective particle dissolution. This cross-linked network seems to rapidly increase as the storage time and temperature increases. Mimouni, Deeth, Whittaker, Gidley, and Bhandari (2010a) compared rehydrated fresh MPC with 2-month-old powder and found a similar result such as a more compact structure, and also a presence of extra surface skin layer of casein micelle in the aged MPC when observed using field emission SEM.

In addition to the powder's components mentioned above, pH and temperature of the solution also influence the solubility of the dairy powders. The solubility of the dairy powders can be improved by rehydration conditions such as temperature, time and shear. Several authors (Crowley et al., 2016; Jeantet, Schuck, Six, Andre, & Delaplace, 2010; Richard et al., 2013) observed a decrease in the amount of sediment or powder rehydration time which seemed to be associated with an increase in stirring speed, mixing time and temperature. However, Jeantet et al. (2010) observed that increasing temperature was more effective at increasing rehydration compared to increasing stirring speed: they found that a 4°C increase, between the range of 26°C and 30°C in mixing temperature reduces rehydration time while doubling the stirring speed from 400 to 800 rpm had the same influence on rehydration time. This finding was in agreement with Richard et al. (2013). From this perspective, different mixing temperatures at two hydration times of constant stirring speed has been used in this study to understand the solubility nature of MCC. A knowledge gap in the literature identified through this review is that though MCC rehydration has been studied with a focus on temperature and mixing conditions, the impact of mixing temperature with respect to the age of the powder is very limited.

2.7.1 Different Approaches to Improve Solubility

The literature includes several contradictory reports that discuss the issue of MCC solubility. Chandrapala, Martin, Kentish, and Ashokkumar (2014) reported MCC solubility can be

improved by high shear methods such as most effective high-pressure homogenization without affecting the structure or mineral balance of casein micelles but however, its effect on age of the MCC powder stored at various temperature was not studied. On the other hand, Schuck et al. (2002) studied the effects of the addition of mineral salts before spray-drying or addition to MCC powder during reconstitution and found that solubility could be improved. Regardless of incorporation mode, the addition of NaCl improved water hydration and decreased rehydration time of casein suspension. This phenomenon is explained by the hygroscopic nature of the salt rather than analysing the micellar structural variation. However structural aspect of casein micelles with respect to NaCl addition was studied by Hussain, Gaiani, Aberkane, Ghanbaja, and Scher (2011). The study reported improved solubilisation of calcium and phosphate from the casein micelle, suggesting dissociation of the micelle happens and results in lumpy, disintegrated protein aggregates with additional secondary structural modification.

Similarly, this finding was in agreement with de Kort, Minor, Snoeren, van Hooijdonk, and van der Linden (2011) study, in which the addition of calcium-chelating agents such as phosphate and citrate ions improved water transfer and decreased rehydration time. This related to the destruction of casein micelle structure such as calcium and phosphate released from micelle and also swelling and dissociation of micelle were observed. Further, the addition of $CaCl_2$ significantly affected micelle organisation by binding of calcium to casein micelle and contributed to the formation of insoluble aggregates. This resulted in a high insolubility index and rehydration time on MCC. In addition to modifying the reconstitution conditions, and the composition of the powder via additives. Solubility can also be improved by optimising process conditions: powder reconstitution can be greatly enhanced by decreasing the inlet and outlet air-drying temperature (Hussain, Gaiani, & Scher, 2012). At present, there is no method available to improve MCC solubility while preserving concentrated and unmodified casein micelles without the loss of calcium or the addition of other substances.

2.7.3 Methods Used for Solubility Test

Previous research on the solubility of milk protein powder have investigated a range of parameters utilising a myriad of different methods. However, even when the approaches use the same standard, there are inconsistences in the data as each study differs with various parameters such as protein concentration, solvent temperature and centrifugation speed or time which makes it difficult to equate the findings from the various studies. Generally, most of the researchers report that during reconstitution of milk protein powder at room temperature, a proportion of the solids remain undissolved, settling out under gravity or centrifugal conditions,

and is referred to as “insoluble material” of the protein powder. However, the centrifugation speed has a large impact on estimates of insolubility. Havea (2006) notes that under certain experimental conditions such as high centrifugation speed, solubilised aggregated proteins may settle and therefore categorised as insoluble. Also, the author inferred that the usage of the term “solubility” should be considered subjective and were relevant only under certain specific experimental conditions. The study determined MPC solubility through centrifugation technique which separated the solutions into two phases; the soluble protein in the supernatant and insoluble sediment as the pellet at the bottom.

In contrast to centrifugation methods that define solubility in terms of total solids, the AOCS (1998) define a method to measure solubility termed the Protein Dispersibility Index. This method is used to quantify protein solubility in which the percentage of protein concentration in the supernatant after centrifugation is compared to the total protein level in the initial reconstituted solution under defined conditions. A drawback of this method is that it is considered to be time-consuming. Another popular method utilised by researchers, especially to study MCC, is nuclear magnetic resonance (NMR) transverse relaxation. This technique provides an insight into the water absorption rate and dissolution of powder particles, thereby displaying powder dissolution kinetics. Regardless of this advantage, a major disadvantage is that a solubility index cannot be quantified as an exact value using this method or does not explicitly indicate whether a milk powder has dissolved/dispersed in solution completely (Fang, Selomulya, & Chen, 2007). Lately, the Malvern MasterSizer particle sizing instrument, which relies on the principle of static light scattering, has been used to determine complete solubilisation. By characterising the hydration state when the particle size distribution falls within a given size range. While this method allows the experimenter to determine if the final reconstituted particle size has been achieved (i.e., approximately 100-500 nm for casein micelles), the results can be biased because of the need to dilute the sample, and thus potentially change the solubility kinetics, prior to the actual measurement of the solution (Chandrapala et al., 2014; Crowley et al., 2016; Richard et al., 2013).

Based on the literature study, a combination of methods to measure the solubility of MCC protein powder gives a good insight to understand the protein characteristics. With respect to Havea (2006) study, the relative term “solubility” is defined in this study as material that is non-sedimentable at the low centrifugation speed and minimum time. Considering the pros and cons of the methods used to measure solubility, a quantitative value can be derived using protein dispersibility index and particle size distribution analysis using Malvern MasterSizer

will also be used in this study to measure MCC solubility characteristics for samples stored for 6-months at different temperatures (20º, 30º & 40º C). In addition, to gain a better insight of the solubilisation process and factors affecting solubility, the powder's microstructure will also be analysed using a combination of scanning and transmission electron microscopy (Gaiani, Schuck, Scher, Desobry, & Banon, 2007; Hussain et al., 2011; Mimouni et al., 2010a).

2.8 Rennet Gelation

In cheese manufacture, milk coagulation is a crucial process and has a significant impact on cheese's chemical and functional properties. Also, this is the foremost step that happens in three phases by the action of enzyme, chymosin which is illustrated in Figure 2.8. In the first phase of enzymatic proteolysis, the chymosin specifically hydrolyses at phenylalanine$_{105}$-methionine$_{106}$ amino bond which is located between para-κ-casein and GMP moieties of κ-casein. Hydrophobic para-κ-casein will remain attached to micelle but the GMP which is negatively charged, hydrophilic and soluble will be hydrolysed and released into whey. The primary and secondary phases of gelation overlap because the aggregation process starts before the complete enzymatic hydrolysis is done. As GMP is released, it results in the reduction of zeta potential, which leads to a decrease in repulsive force and hydrophobic interactions will be increased. Thus, in the presence of calcium ions, which further reduce repulsive inter-micelle forces, destabilised casein micelles start to aggregate. Initially, small linear chains are formed which gradually aggregate to form clumps, clusters and finally converted into three-dimensional gel-networks. In the final phase of gel formation, the fat globules are trapped inside and whey is expelled outside as a result of syneresis due to the stronger gel network being formed (Thompson et al., 2009).

Figure 2.8 Casein micelles coagulation process by the action of chymosin (Heino, 2010).

2.8.1 Factors Affecting Rennet Coagulation

The milk coagulation is a complex process and influenced by multiple factors. It is noteworthy to understand these effects on rennet coagulation as it strongly affects final cheese quality and yield. The various compositional and environmental factors that affect rennet coagulation are milk protein and fat concentration, pasteurization temperature, cooling and cold storage of milk, milk homogenization, coagulation temperature, pH, calcium chloride and rennet concentration. Additionally, it may also be influenced on-farm variables such as lactation stage, diet, and the heath of the cow. These effects are likely to be more noticeable in New Zealand, where milk is predominantly from pasture-fed spring-calving herds (Fox, Guinee, Cogan, & McSweeney, 2017). Out of all these factors, the most prominent ones are pH, CaCl2 content, temperature, rennet level and milk protein concentration which will be discussed in detail below.

An important aspect of characterising a milk protein system's rennetability is determining the time it takes for the system to the gel and gel strength. The rennet coagulation time (RCT) is defined as the time taken for the formation of gel from the addition of rennet in the system (sol-to-gel process). It gives a very good index of the milk's gelation potential and low RCT is correlated with good gel formation and high curd tension. Gel (curd) strength is a term that can be used interchangeably with gel (curd) firmness, curd tension (CT) and defined as the force required to create a given deformation or strain. There is an inverse relationship between RCT

and CT in general, and thus any factor that reduces RCT increases CT and vice versa (Fox et al., 2017).

2.8.1.1 Effect of pH

The coagulation time of rennet induced coagulation increases with increasing pH specifically beyond pH 6.4 because of the pH effect on the activity of the enzyme. The reduction of pH from 6.8 to 6.0 (Daviau, Famelart, Pierre, Goudédranche, & Maubois, 2000; Nájera, De Renobales, & Barron, 2003) increases the aggregation of destabilised casein micelles and results in a shorter coagulation time with higher curd firmness. Fox et al. (2017) noted that curd firmness increases with decreasing pH to reach a maximum at 5.9-6.0. Lowering the pH below the range of 5.9, decreased the CT but this might be because of colloidal calcium phosphate (CCP) solubilisation. Rennet gelation studies by (Cheryan, Van Wyk, Olson, & Richardson, 1975; Nájera et al., 2003) also reported decreases RCT and CT with decreases in pH.

2.8.1.2 Effect of Coagulation Temperature

The primary benefit of coagulation temperature is on the secondary, non-enzymatic phase. Coagulation of rennetted casein micelles is not significant below 18ºC. Above this temperature and till 40-45ºC, coagulation time decreases. Above 45ºC, the coagulation rate increases again however there is an overall decrease due to a decrease in the primary phase resulting from enzyme denaturation. The most optimal temperature for rennet coagulation in cheese manufacture is in the range of 27-32ºC because it favours the growth of mesophilic starter bacteria and also improves the coagulum structure. However, increasing temperature in the range of 18-45ºC is associated with lower RCT and higher CT (Carlson, Hill, & Olson, 1986; Cheryan et al., 1975; Nájera et al., 2003). The attribute (Kowalchyk & Olson, 1977) behind the strong effect of temperature on aggregation has been hypothesised to be due to hydrophobic interactions, which enhances and increases coagulation rate with increasing temperatures. Carlson et al. (1986) reported another theory of higher temperatures, noting that increasing temperature favours reduced Ca^{2+}solubility and increased CCP levels can also promote gel coagulation.

2.8.1.3 Effect of Added Calcium

The primary benefit of Ca^{2+} concentration is on the secondary rennet coagulation phase. A critical calcium concentration is required for coagulation to occur and calcium increases above this critical concentration result in, RCT decreases and increasing CT (Carlson et al., 1986; Nájera et al., 2003). Also, added $CaCl_2$ favours three advantages i.e., an increase in the

concentration of Ca^{2+} and CCP and decreases pH. A study by McMahon, Brown, Richardson, and Ernstrom (1984) reported 50mM calcium level reached the minimum RCT and in contrast 10mM calcium level reached maximum CT as measured by a Formagraph. This result is similar to a Udabage, McKinnon, and Augustin (2001) study, tested using oscillatory shear strain-based dynamic rheology. Fox et al. (2017) proposed the reason for CT decreasing at higher calcium levels, might be due to the interaction of negatively charged carboxyl groups on para-casein interacting with excess Ca^{2+} levels to decrease the residual negative repulsive charge on the casein, making it more prone to aggregation.

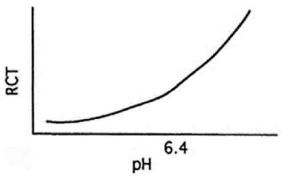
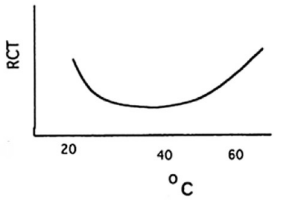
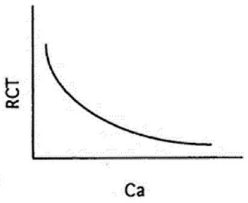
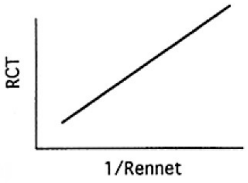
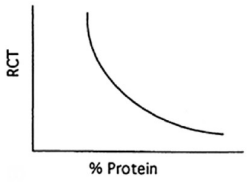
2.8.1.4 Effect of Rennet Level

The primary enzymatic rennet coagulation phase is directly dependent on the amount of rennet added and there is a linear relationship between an inverse of rennet level and RCT. The literature findings, however, vary in relation to the effect of rennet level on CT, some showing no effect or minor reduction and in contrast, some studies show an increase in CT depending on the lactation stage. The gel strength is highly determined by the rennet source; microbial rennet has a minimal effect on gel strength in comparison to animal rennet (Fox et al., 2017).

2.8.1.5 Effect of Milk Protein Concentration

Historically, increasing protein content in concentrated milk resulted in faster coagulation time, RCT with increasing κ-casein hydrolysis rate and CT (Daviau et al., 2000; Guinee, O'Callaghan, Pudja, & O'Brien, 1996; Sandra, Cooper, Alexander, & Corredig, 2011). The difference between using low and high concentration factor-based milk for rennet coagulation tested by Dalgleish (1980) and found the rapid increase in aggregation step results in enzymatic hydrolysis of κ-casein as the rate-limiting step using high concentration factor. But, the coagulation time is dominated by the casein aggregation step when the low concentration factor used. Similiarly, Orme (2000) also observed that increased UF concentration is related to a decreased percentage of hydrolysed κ-casein and small cluster formation with less reactivity, leading to decreased whey expulsion and rough cheese texture.

Table 2.2 Impact of various factors on rennet coagulation time (Fox et al., 2017).

Factor	First Phase	Second Phase	Graphic Represenation of Overall Effect
pH	+++	-	
Coagulation Temperature	+	++	
Added Ca	-	+++	
Rennet Concentration	++++	-	
Protein Concentration	+	++++	

2.8.2 Recent Research Using Microfiltrated Milk for Cheese Manufacture

Acharya and Mistry (2004) reported that cheese milk concentration using ultrafiltration or evaporation results in low-moisture cheese with decreased meltability and slower proteolysis during cheese maturation stage. The decrease in the proteolysis rate suggested being due to their proteolytic enzyme resistance and also inhibition by whey proteins (Bech, 1993). Mistry

and Maubois (1993) reported that ultrafiltration had no significant adverse effect on the cheese maturation. On the other hand, Hinrichs (2001) observed crumby texture and bitter taste. Even though UF concentrated cheese milk considered to be economically beneficial, the study by different author reports the taste, structural and functional properties of the cheese are compromised. The decrease in proteolytic effect also been observed when microfiltration has been used for the protein standardisation method by increasing native whey protein content of the cheese milk (St-Gelais, Piette, & Belanger, 1995).

Similarly, Neocleous, Barbano, and Rudan (2002b) suspects three factors might be responsible for the decreased proteolysis in MF cheese during maturation are:-

- The reduced amount of substrate (casein) availability for chymosin due to low moisture in the non-fat substance (NFS). This effect has specifically noted when the concentration factor of MF increased.
- When using 0.1 μm membrane-based MF, an increased concentration of high-molecular weight α_2-macroglobulin (serum protein) retained in the retentate. Similarly, Jost, Brandsma, and Rizvi (1999) also noted the presence of HMW-serum protein in the MF retentate.
- MF-based cheese has lower residual rennet content because chymosin dosage can be reduced due to a faster coagulation rate.

However, Rodríguez, Requena, Fontecha, Goudédranche, and Juárez (1999) compared the cheese texture and flavour of MF and UF produced cheese, cheese hardness increases in MF cheese when using higher casein content milk for coagulation as availability of whey protein-casein linkage was low. Despite that the development of aroma and flavour in MF cheeses is not affected, unlike in the case of UF cheeses, where higher content of whey proteins was present (Bech, 1993).

From a different perspective, MF is found to be the most preferred method for bacterial reduction of cheese milk relative to pasteurisation for reduction in non-starter bacteria and spores. This is because thermal methods to reduce non-starter bacterial load result in whey protein denaturation and thus, weakens milk coagulation properties. Hence, Maubois (2002) concludes that microfiltered casein concentrate is a promising substitute for cheese production.

Thomet and Gallmann (2003) highlighted using the MF-based cheese manufacturing methods increased the yield of cheese by 3 to 5% while also reducing the production costs. But in contrast, Papadatos et al. (2003) mentioned microfiltration increased production costs but also

increased net profits. Still, the economic value of microfiltration remains uncertain. Govindasamy-Lucey, Jaeggi, Johnson, Wang, and Lucey (2007) reported MF retentate-based cheese has 2-3% less moisture content in comparison to part-skim milk-based cheese.

Although most researchers mention that microfiltration methods are very effective in cheese-making, very limited research data is available utilising microfiltration retentate in either liquid form (Brandsma & Rizvi, 2001; Govindasamy-Lucey et al., 2007; Neocleous, Barbano, & Rudan, 2002a; Neocleous et al., 2002b; St-Gelais et al., 1995) or powder form (Simov, Maubois, Garem, & Camier, 2005). Together these studies provide important insights that the use of MF retentate has higher pH, reduced moisture content, higher hardness and decreased proteolysis during aging.

2.8.3 Methods used to measure Rennet Gelation Characteristics

The milk coagulation and gel-forming characteristics are measured by the parameters of gelation time, gel strength and gel firming rate using various methods. These parameters play a significant role in optimising the production of large-scale fermented dairy products. Among the dynamic gel firmness tests, the Formagraph and the low-amplitude oscillation rheometry (LAOR) will be used in this study. As the former one, although being a semi-destructive test, has the benefit of enabling the measurement of many samples at once, while the latter one results in more detailed data and is a non-destructive milk test but is limited by only measuring one sample at a time.

2.8.3.1 Formagraph

Prior to the development of LAOR instruments, the Formagraph was the most popular dynamic measuring instrument used to measure the rennet gelation properties of the milk. The working principle of the Formagraph was reviewed in detail by Fox et al. (2017). The instrument contains a metal heating block with ten cuvettes where milk samples oscillate. A pendulum loop, which is present in each cuvette, records the milk sample's viscosity. At the start of the experiment, a straight line is formed due to less viscous milk samples, and also the pendulum remains at its original vertical zero position. Once the gel formed, the viscosity of the sample increases, and the pendulum loop is dragged from its vertical position through the movement of the samples, which results in line bifurcation as shown in Figure 2.9. The milk coagulation curves are electronically captured and shown on a computer display. The final output data after the given renneting time also saved as files in the software that may be used to calculate the rennet coagulation parameters. The parameters that can be derived using these files are;

- Rennet Clotting time (RCT), r (min), i.e., the time taken for the formation of gel from rennet addition.
- k20 (min), i.e., the time from the start of gel formation until a width of 20mm is reached. This also indicates the optimal cutting time of the gel. The curd firming rate (CFR) can be calculated by 1/k20.
- a_{30} (mm), i.e., the curd firmness after rennet addition of 30 minute which is the width of the curve.

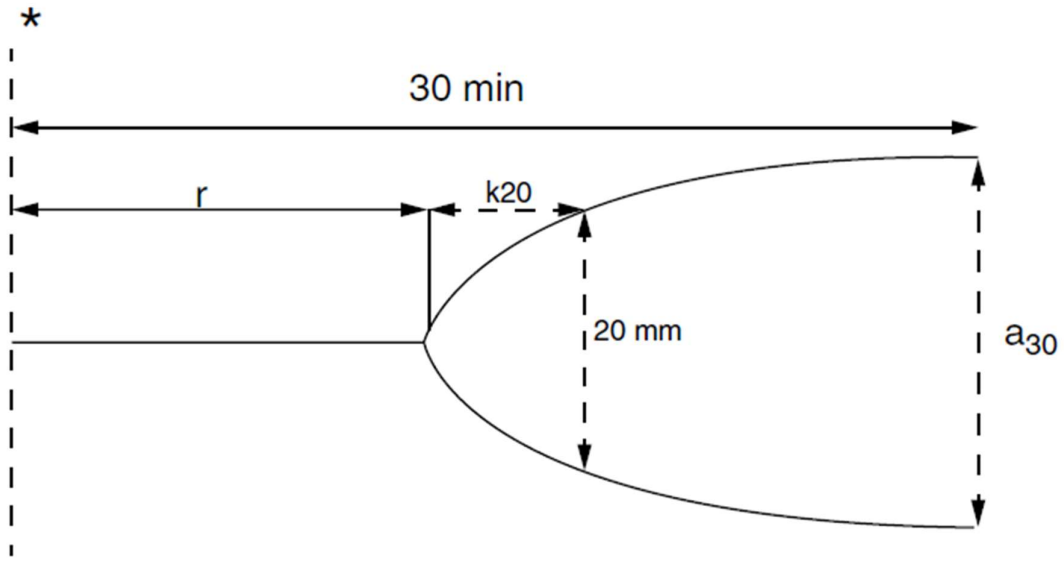


Figure 2.9 The Formagraph monitored typical rennet coagulation curve after 30 Minute.*-Rennet addition point (Fox et al., 2017).

A milk sample with a strong coagulation potential takes a limited time to coagulate (low r-value) and attains higher gel strength (high a_{30} value) and curd firming rate (low k20value). Formagraph offers a great advantage of measuring ten samples at a time (Fox et al., 2017).

2.8.3.2 Low-Amplitude Oscillation Rheometry (LAOR) method

Controlled strain or stress rheometers are increasingly being used as a research tool for the continuous measurement of the linear viscoelastic (LVE) properties of milk gelation as a function of time from rennet addition. Milk gelation is measured using bob-cup geometry (Figure 2.10a), in which enzymatically modified milk sample is added and inserted into the rheometer's temperature-controlled measurement cell. After bob insertion, it oscillates at a very low-amplitude ("amplitude refers to the maximum displacement of any point on the oscillating cup (and hence in the milk sample or on the inner bob) from its mean, or 'zero', position (Fox et al., 2017)"). Foegeding, Vardhanabhuti, and Yang (2011) stated LAOR is a non-destructive

measurement because the formed gel structure is not destroyed by defining strain value below the upper limit of the LVE region range.

Using LAOR (Figure 2.10b), the following rennet gelation parameters can be derived from the curve of storage modulus (G') and loss modulus (G") versus time, i.e., gelation time (GT, min), gel strength/firmness (Pa) after a given renneting time, maximum gel firming rate (GFR, Pa/min). G' represents the magnitude of energy stored and G" represents the energy loss per the cycle of deformation. At the start of the gelation process, the viscous behaviour of the sample dominates (G" > G') and in the middle of the gelation process, the elastic nature of the sample dominates (G' >> G"). The increase in G' represents gel strength and the number of bonds in the gel network (Foegeding et al., 2011).

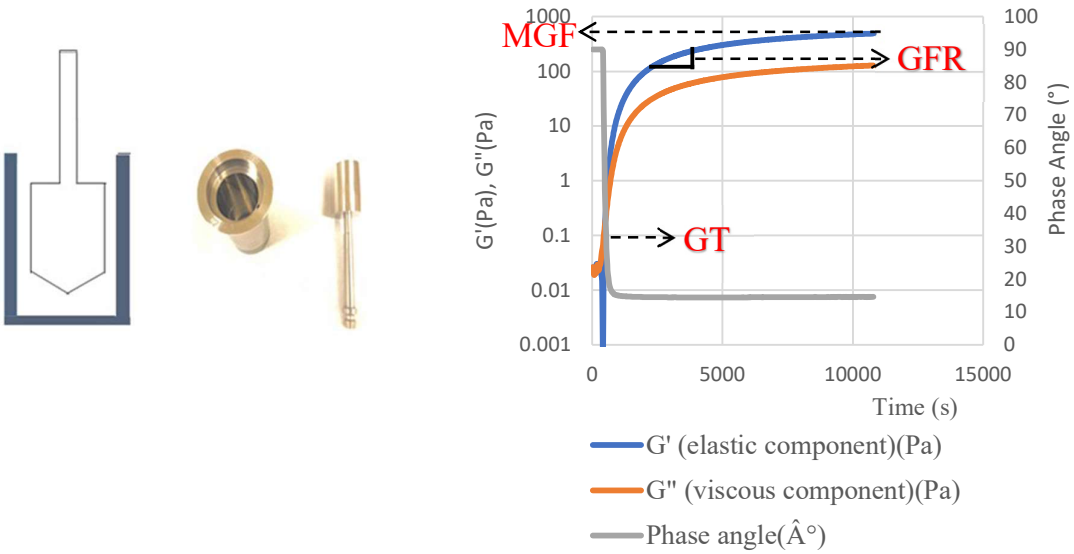


Figure 2.10 (a) Bob and cup geometry-based measurement system (C25- specifies a diameter of 25mm bob in a diameter of 27mm cup).

(b) Rennet coagulation pattern of milk samples using rheometry

GT – Gelation Time; MGF – Maximum Gel Firmness; GFR – Maximum slope of G′ vs time; SCT20Pa – Set-to-cut time at a suitable firmness (20Pa).

GT has been expressed differently by different authors. Fox et al. (2017) defined GT as the time when a threshold value of 0.2 Pa attained by G'. Others described GT as the time at which phase angle (δ) was 45º or the intersection between G' and G" on the curve (G'=G"). Finally,

GT was defined as the point where G'=1 by a few other authors. GFR represents the maximum slope of G'-time curve. A milk sample with a strong coagulation potential takes a limited time to coagulate and attains higher gel strength. LAOR is reliable and accurate for the measurement of rennet coagulation properties however, only one sample can be analysed at a time and considered to be more sensitive to minor changes in the sample.

3. Research Hypothesis

3.1 Research Aim

Examining the solubility and rennet gelation characteristics of micellar casein concentrate (MCC) powder owing to the shelf-stability analysis in cheese manufacturing.

3.2 Research Objectives

1. To determine the solubility of fresh MCC powder and also for the stored powders at different temperatures of 20°, 30° & 40°C for the total period of 6 months using a combination of methods such as moisture dish and static light scattering analysis.
2. To examine the colour changes of MCC with respect to storage time and storage temperature and also visualise the structural variation of MCC powder using combined SEM & TEM microscopic techniques.
3. To find the solution for the improvement of insoluble material i.e., enhancing the solubility of MCC by utilising single-stage homogenization method.
4. To assess the rennet gelation characteristics of MCC powder, the rheometric and Formagraph analysis was compared and carried out to derive variables of the rennet coagulation properties for the application of cheese production.
5. To understand the impact of hydration time on MCC in the prepared low heat-skim milk powder (LH-SMP) base and also to study the effect of renneting set temperature and pH on the rennet gelation properties, 24 hr hydration and 3 hr hydration time was studied with respect to solubility characteristics.
6. To evaluate the variation of gelation time and gel strength of the stored samples in comparison to fresh sample using Formagraph analysis.

3.3. Research Significance

In New Zealand, the dairy industry is very important and increases the country's trade by providing high profit through exporting dairy products. To meet the consumer demands and deliver the best to the world, MCC has high market potential with numerous benefits for the dairy industry. By understanding the functional properties through this research will highlight the key benefits that industry can attain by manufacturing the product, which in turn increases country's revenue.

It is important to understand the challenges that customers may face on storing the product for long period of time. On this front, the study verifies the functional properties for product's shelf-life and provides an insight on functional changes with respect to the storage conditions.

Overall, this study will assist dairy product developers to understand functional properties and challenges that could be faced with long-term storage, which will also aid them in good manufacturing practices for the nutritional dairy ingredients.

4. Materials and Methods

4.1 Materials

4.1.1 Micellar Casein Concentrate

The commercial micellar casein concentrate (MCC) powder of 20kg bag was purchased from Leprino Foods Dairy Product Company (USA), manufactured on 21st Feb 2019. It should be noted that the experimental testing started on the powder from August 2019 and until this time the MCC powder was stored at room temperature on arrival at Massey University. The time and temperature storage conditions from manufacture and during shipping until arrival at Massey University are unknown. The microbial analysis conducted by routine factory results for the used MCC bag was within specifications. Table 4.1 explains the basic compositions of commercial MCC powder provided by the manufactures.

Table 4.1 Basic compositions of MCC powder used in this study.

Components	MCC (% w/w)
Total Protein (dry basis)	86.0
Fat	3.0
Ash	7.5
Moisture	7.5
Casein-to-Whey Ratio	95:5

4.1.2 Low Heat-Skim Milk Powder

The commercial low heat-skim milk powder (LH-SMP) was used as the base milk to test the renneting properties of skim milk fortified with MCC. The LH-SMP was acquired from New Zealand Milk Products, New Zealand in the product name of Skimmilk powder - Regular, Low heat and compositions are outlined in Table 4.2.

Table 4.2 Compositions of LH-SMP used in this study.

Components	MCC (% w/w)
Total Protein (dry basis)	32.9
Fat	0.9
Ash	7.9
Moisture	3.8
Lactose	54.5

4.1.3 Rennet Source

The natural calf rennet liquid was purchased from RENCO, Eltham, New Zealand and the batch number specified for the product was 4902010 respectively. The rennet enzyme strength was 288 IMCU/mL and the microbial analysis conducted by routine factory results was within specifications. For dilution of rennet, Milli-Q water has been used.

4.1.4 Chemical Reagents

In some experiments, water purified by reverse osmosis was used whereas in others, double deionised Milli-Q water, which filtered using Milli-Q apparatus was used. Hydrochloric acid (HCl) was of a technical reagent grade used in this study for adjusting pH of the solution.

4.2 Sample preparation

4.2.1 Storage of MCC Powders

From the time of the study started, the MCC powder used in this study was frozen to -18°C as a reference sample. As a study focused on the storage stability of the powder, the MCC samples were stored at three different temperatures of 20°C, 30°C and 40°C for 6 months (180 days). As earlier studies stated a fast solubility deterioration occurs at 40°C, samples were tested every week (7 days) and for 30°C stored sample, testing was done fortnightly (15 days) As 20°C considered to be room temperature, testing was done for every 2 months (60 days).

In airtight aluminium foil bags, the individual samples were tightly sealed and placed in heated rooms that were maintained at above-mentioned temperatures. The difference in the powder properties stored under these conditions was tracked to evaluate the effect of storage temperature on MCC properties over a prolonged period of time using the methods listed in the following sections. After storage, samples were frozen to -18°C for further testing.

4.2.2 Reconstitution of MCC Powder for Solubility Testing

The reconstituted MCC solution was prepared using 3.5% protein (wt/wt %) in RO water solution. The protein content is 86% per 100g of MCC powder according to the manufacturer's compositional analysis. The mass of water and MCC powder was calculated to give the final solution to the desired concentration. The water solution was weighted and heated in a stainless-steel beaker of the desired temperature in a hot plate. In the meantime, the solution temperature was well-set in the water bath to maintain the same temperature throughout the experiment. For stirring the solution, the flat propeller designed for dissolving powders

attached to an electric overhead stirrer was utilised and speed has been set to achieve an adequate stirring for powder wetting.

The MCC powder was slowly added with a teaspoon by giving the proper interval for the complete dissolving of the powder between each addition. The total mass of the powder was added in a total period of 3 minute. During powder addition, the stirring speed was set high around 700-750 rpm for wetting. Once all the powder has been added, the stirring speed was decreased and set constant of 350 rpm throughout the remaining stirring time. The stainless-steel beaker with the solution was covered with tin foil to avoid evaporation loss. During powder addition and stirring time, plenty of foam was observed in the solution. The certain amount of homogenous solution was taken out once the hydration time of 30 min has been reached and stirring process has been continued for the total hydration time of 3 hr before tests were commenced.

The solubility determination at different temperature of 25, 30, 40, 50 and 60°C with two different hydration times of 30 min and 180 min was carried out. The shortest and longest period of hydration time were selected to reflect typically industrial use.

4.2.3 Reconstitution of Skim Milk Base

Base skim milk solutions were prepared by reconstituting skim milk powder to 10% (w/w) total solids in RO water. The mixture was covered with tin foil to minimise evaporation during reconstitution and stirred at the temperature of 50°C for 30 min to ensure complete dissolution. Preliminary studies showed 100% solubility of LH-SMP solutions at this temperature and stirring time.

4.2.4 Reconstitution of Skim Milk-Fortified MCC for Rennet Gelation

To understand the rennet gelation property of MCC, the total protein content used in this study is 5.6%. The milk's protein content of 3.5% skim milk powder and for the remaining 2.1%, MCC powder was utilised as cheese milk extender. As the next step of reconstituting skim milk-MCC solution, the question arose and interested to find how hydration time of MCC impacts the rennet gelation property. So, the study tested 24 hr versus 3 hr hydration time of MCC in the skim milk base.

In 24hr hydration time, the skim milk and MCC were added together in RO water and stirred at 50°C temperature for 30 min. A few grams of samples removed for solubility testing using moisture dish and particle size analysis. Then, the remaining samples were kept at 4°C refrigerator to allow 24 hr equilibration time. Before commencing rennet gelation experiments,

the samples were removed from the refrigeration and allowed to equilibrate at room temperature.

In 3 hr hydration time, the skim milk was reconstituted at 50°C for 30 min and then left at the refrigeration of 4°C for the equilibration time of ~20 hr. Then, the samples were removed and heated to 50°C using a hot plate for adding MCC powder to the base solution. Then, ~2.1% protein content of MCC were added to the base solution at 50°C for 30 min and then, a few grams of samples removed for solubility testing using moisture dish and particle size analysis. The remaining samples were allowed for equilibration time of 3 hr in the 4°C refrigerator before the commencement of rennet gelation property analysis.

4.2.5 Rennet Preparation

The liquid rennet was diluted was in the ratio of 1:20,000 using Milli-Q water. For rheometric analysis, rennet diluted in the ratio of 1:10 using Milli-Q water and then 100 µl of diluted rennet was added to 50 ml of MCC-fortified skim milk solution. For Formagraph analysis, rennet diluted in the ratio of 1:100 using Milli-Q water and then 200 µl of diluted rennet was added to 10ml of MCC-fortified skim milk solution. The final enzyme strength of the complete milk used in both the Formagraph and rheometric analysis was 0.00576 IMCU/ml. Once diluted, the rennet stored at 4°C refrigeration for two days maximum and then discarded.

4.3 Experimental Methods

4.3.1 Solubility Determination Using Moisture Dish Analysis

The method used in this study was adapted from Carr et al. (2004). In two individual pre-weighed moisture dishes, approximately 2 g of sample was placed using Pasteur pipette and the total weight of moisture dishes along with the bulk solution was noted. Then, the moisture dishes were placed in the steam bath of 100°C to avoid spattering of the solution in the forced oven (Conthem, New Zealand). Since the total sample weight was less, it took around 10 min to dry the liquid solution in the steam bath. Immediately, the moisture dishes were placed in the forced oven for drying at 108°C overnight.

The remaining sample was centrifuged using ThermoFisher Scientific Multifuge X1R (Germany) at 700g for 10 min in a 50 ml centrifuge tube. The centrifugal force helps to sediment the insoluble particles as pellet while soluble particles remain in the supernatant. Following 10 min of centrifugation, approximately 2 g of supernatant was placed in the two pre-weighed moisture dishes using Pasteur pipette as described above for the total solid analysis. After drying, the moisture dishes were removed from the oven and allowed for

cooling to room temperature, which took approximately 20 min in a desiccator containing dry silica gel to avoid condensation and then weighed. The same procedure was repeated for the stirring time of 30 minute and then for 180 minutes.

The total moisture of the sample was calculated using the equation below,

$$\%\, Total\ Moisture = \frac{w2-w3}{w2-w1} * 100 \qquad \text{Equation 1}$$

Where w1 - weight in grams of moisture dish + lid

w2 - weight in grams of moisture dish + lid + sample (before drying)

w3 - weight in grams of moisture dish + lid + sample (after drying)

$$\%\, Total\ Solids = 100 - \%\, Total\ Moisture \qquad \text{Equation 2}$$

Then, the solubility of each sample at different temperature and stirring time calculated using the equation below,

$$Solubility = \frac{Total\ solids\ of supernatant}{Total\ Solids\ of\ Bulk\ solution} * 100\% \qquad \text{Equation 3}$$

4.3.2 Solubility Determination Using Particle Size Analysis

The particle size distribution (PSD) analysis of MCC suspension after the dispersion of 30 min and 180 min was carried out using Malvern Mastersizer 3000 (Malvern instruments Ltd. Worcestershire, UK). During analysis, the particle refractive index of casein micelles was set to 1.57 (Griffin & Griffin, 1985) with an absorption index of 0.001 and dispersant (water) refractive index of 1.33. Before each measurement, a cleaning cycle was run and the resulting data are noted as volume-based particle size distribution based on the average of duplicate measurements. The measurements were done by adding samples dropwise into the dispersing unit of the instrument until a laser obscuration was achieved.

4.3.3 Powder Colour Analysis

For stored powders, the colour measurements were carried out using Chroma Meter CR-400 (Konica Minolta Business Technologies, Inc., Tokyo, Japan) owing to the colour coordinates L* (white = +, black = -), a* (red = +, green = -) and b* (yellow = +, blue= -) space. Before measurements, the L*, a* and b* values were standardised using the white calibration plate. Then, the powder was dispersed in a small circular container and placed above the colorimeter

for a total of three measurements. The results expressed as an average of three by following the method of Nasser et al. (2017) study.

The browning index (BI) was determined from the formula (equation 4) integrating the L*, a* and b* values to determine the intensity of the brown colour of the samples.

$$\text{Browning Index, } BI = \frac{100.\left[\left(\frac{a*+1.75XL*}{5.647XL*+a*-3.012Xb*}\right)-0.31\right]}{0.17} \qquad \text{Equation 4}$$

4.3.4 Scanning Electron Microscopic Analysis

The MCC powder microstructure was imaged using scanning electron microscopy according to McKenna, Lloyd, Munro, and Singh (1999). A double-sided tape was attached to an aluminium stub, which helps a thin layer of powder particles to be mounted on the stub. As a pre-treatment step, samples were coated by ion sputtering with 20 nm of gold. Then, the images were examined under the Cambridge 250 Stereoscan scanning electron microscope (Cambridge Ltd, Cambridge, England) at appropriate magnifications.

4.3.5 Transmission Electron Microscopic Analysis

The fresh and stored MCC powders were dispersed into propylene glycol and then combined with a 1:1 mixture of warm 3% low-temperature gelling agarose following the method of McKenna et al. (1999). The mixed solution was transferred to a microscopic slide, left to settle down and then cut into 1 mm^3 pieces. The chopped blocks were placed in a bijou bottle containing 3% glutaraldehyde/cacodylate solution for 2 hours. The cubes were washed for 30 min and 1 hr with cacodylate buffer and left for 1 hr at room temperature. Then, cubes were placed with 1 ml of a 50% osmium tetroxide (2% liquid) and 50% cacodylate/HCl buffer containing solution. After 2 hours, transferred to 1 ml of 1% uranium acetate for 30 minute and washed with water before dehydration. Dehydration washing step using 50, 70, 90 and 100% ethanol for 5, 30, 30, and 180 min respectively. After discarding ethanol, the bottle was filled with 20 ml of epoxy resin and dodecyl succinic anhydride with a drop of dibutyl phthalate comprised incomplete resin and left on a rotator overnight. Then, blocks were transferred to complete resin for further rotation for 4 hours. In each of three moulds containing fresh complete resin, one sample cube was added and then baked overnight at 60°C.

Using a Reichert Ultracut microtome, the prepared samples were microtomed and mounted on 3 mm copper grids for staining with lead citrate. The grids were placed at an accelerating

voltage of 60 kV in a Philips 201 transmission electron microscope (Philips, NL-5600 MD Eindhoven, The Netherlands).

4.3.6 Homogenisation

High-pressure homogenisation was performed to investigate particle size reduction on reconstituted MCC samples as described by Chandrapala et al. (2014). The single-stage homogenisation was performed at 50°C followed by an operating pressure of 100, 150 and 200 bar using a bench-top homogeniser (Homolab 2, FBF Itatlia, Italy). After homogenisation, the sample's solubility rate was determined as described in Section 4.3.1.

4.3.7 Rennet Gelation Test using Rheometric analysis

The rheological properties of the reconstituted MCC samples were analysed using a low-amplitude dynamic oscillation rheometer (Malvern Kinexus, Worcestershire, UK) with a cup-and-bob (25 mm diameter) arrangement according to Ping (2015) study. The viscoelastic properties of the milk gels were made sure to measure within the linear viscoelastic region (LVR) by monitoring gel formation at 30°C with a fixed frequency of 1 Hz and a fixed strain of 0.25 % in a time sweep mode. Before rheometric analysis, the reconstituted MCC samples were removed from the refrigerator and kept in the 30°C maintained water bath to attain the renneting set temperature. Then, 100 µl of 1:10 ratio of freshly diluted rennet solution was added to the 50 ml of reconstituted MCC sample resulting in standardized addition rate of 0.0576 IMCU/mL. The sample solution was stirred for 30 sec and transferred to the rheometer cup and bob was lowered to the measuring position. The rheometric measurements were started after 3 min of temperature equilibration. The time was noted manually using stopwatch from the rennet addition to the rheometric 1st measurement started. A thin layer of mineral oil was added to the sample surface to avoid the evaporation of the sample. Measurements for storage modulus (G′) and loss modulus (G″) were taken every 30 sec for 3 hr. From the obtained G′-G″/t curve, RCT, MGF, MGFR, and SCT parameters can be derived as described in Section 2.8.3.2.

4.3.8 Rennet Gelation Test Using Formagraphic Analysis

The substrate and rennet composition were kept constant to test the influence of temperature and pH over the rennet coagulation properties of MCC sample using a Formagraph (Foss Electric, Hilleroed, Denmark) as described by McMahon and Brown (1982). A 10 mL of each sample solution was added in two wells of sample cuvettes and placed over the service module heating plate. The samples were allowed to attain the target temperature of 30°C and 32°C on

the heating plate and checked using a thermocouple. Then, 200 µl of 1:100 ratio of freshly diluted rennet solution (0.0576 IMCU/mL) was added in the set of spoons which then added to the samples at the same time and stirred well for approximately 15 seconds. The sample cuvettes were immediately placed into the recorder module and measurements were begun. After 30 minutes, the measurements were stopped and the samples were analysed in duplicate. This afforded to give direct measurements of RCT, k20, A30 and CFR parameters as described in the Section 2.8.3.1.

5. Solubility Testing of Fresh and Stored MCC

5.1 Introduction

MCC is often incorporated as an ingredient in many food applications such as increasing the protein content of cheese and yoghurt and also to standardize the protein content of milk. A prior dissolution of MCC powders in water is necessary for most applications to allow the powder to completely convey its functional properties. Therefore, rehydration and solubility are essential end-use properties need to be evaluated for MCC powder. The aim of the current investigation is to understand the effect of hydration time tested under various hydration temperatures on the solubility of MCC. An additional aim is to understand the influence of storage under different temperature conditions on MCC solubility that could be faced in industry.

5.2 Results

Chapter 4 describes the methodology for determining total solids (Section 4.3.1) and particle size (Section 4.3.2) of MCC solutions and all experiments were performed in duplicate. Initially, the solubility tests were performed on MCC powder that had not been exposed to high temperatures and denoted as fresh powder in the study to understand the effect of hydration time and temperature on the solubility of MCC powder. Then, one hydration time has been selected to test stored samples based on our focus of interest to reduce the time for industrial benefit under various hydration temperature. This has been done to understand the effect of storage time and temperature on the solubility of the MCC powder.

5.3 Effect of hydration time and temperature on the solubility of Fresh MCC powder

5.3.1 Using Moisture-Dish Analysis

The solubility determined at various hydration temperature of 25, 30, 35, 40, 50, 60 and 70°C for the hydration time of 30 and 180 minute and individual results can be viewed in Figure 5.1. The solubility of reconstituted MCC solution increased as the hydration temperature and time increased showing both the factors have a strong influence. In the case of 30 min, the maximum solubility was obtained at a reconstitution temperature of 50°C. But, in the case of 180 minute, at the temperature of 30°C solubility increased above 90% and showed the optimum processing window lies between 30-50°C. Overall, from the data it looks by minimizing hydration time we can attain maximum solubility by increasing hydration temperature. As the result, hydration

temperature strongly influence the rehydration and our results were similar to Jeantet et al. (2010).

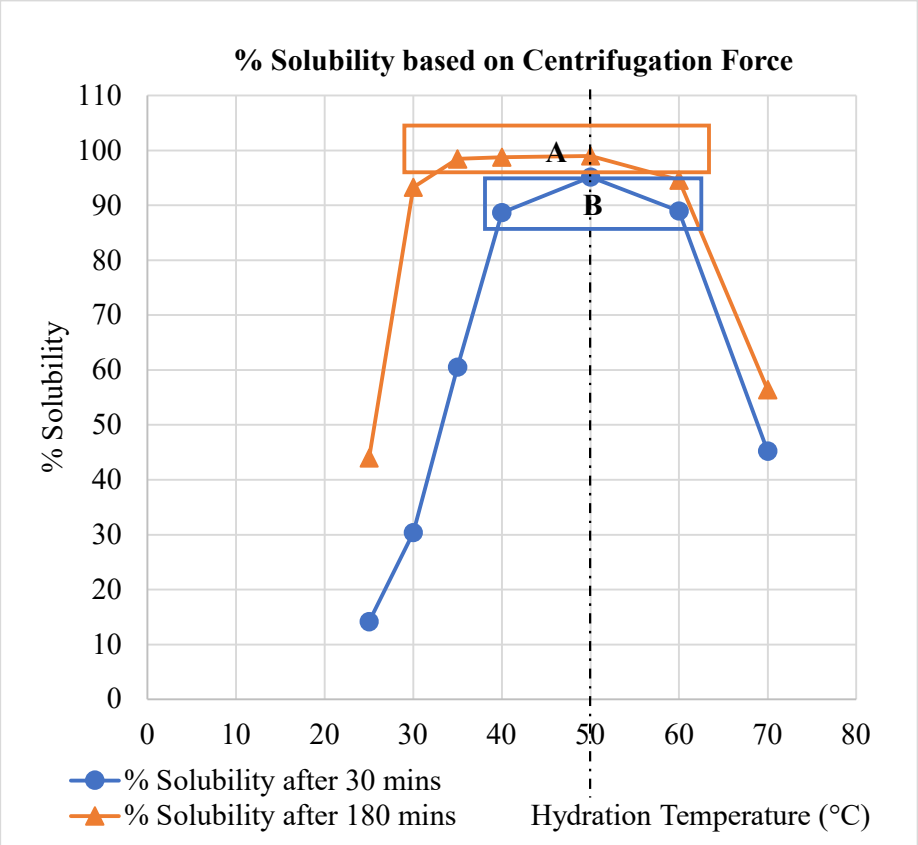


Figure 5.1 Effect of hydration time and temperature on the solubility of fresh MCC powder. Optimal processing windows are shown for A) 180 min hydration and B) 30 min hydration time.

After 50°C, solubility started to drop and tends to show some protein reaction happens. Regardless of hydration time, at the reconstitution temperature of 60 and 70°C, the parallel similar trend has been observed. The possible phenomenon for the decrease in the solubility after the reconstitution temperature of 50°C will be discussed in the following sections.

5.3.2 Using Mastersizer Analysis

To verify the numerical solubility percentage derived using centrifugation force, a particle size distribution has been done on fresh reconstituted MCC bulk solutions and supernatants of different hydration time and temperature. Figure 5.2 shows a typical size distribution, as the particle size of casein micelles falls under the range of 50-500 nm, the complete soluble particles could be indicated as the single peak in the size classes of 0.01-1 μm. In the bulk

solutions regardless of hydration time and temperature, the overall size class distribution had the bimodal distribution with the size classes up to 100 µm. Even though the results of moisture dish analysis predicted 99% numerical value but in contrast PSD analysis indicated still the solutions made up of undissolved large casein particles. The supernatant, here which is the soluble material falls in the expected size classes of 0.01-1 µm.

The powder's rehydrated PSD might be influenced due to particle interaction that occurred during the production process. Bouchoux, Gésan-Guiziou, Pérez, and Cabane (2010) reported casein micelles are well-segregated in their native form so that weak inter-particle interactions occur and do not disrupt the micellar structure. However, hydrated casein micelles are soft structures that react to environmental changes. In specific, the compressive forces such as osmotic stress or the forces applied during concentration by evaporation or filtration process may alter the voluminosity and deform their structure. Thus, the area of the size class observable from 0.01-1 µm was classified as the mixture of altered casein micelles and single caseins.

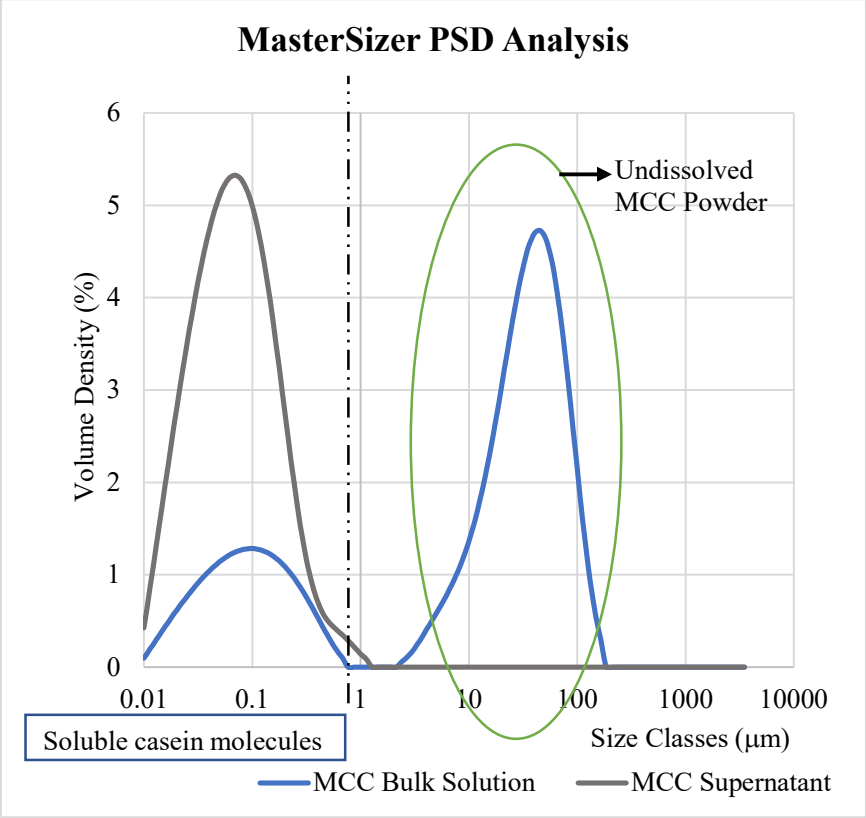


Figure 5.2 Overall Particle Size Distribution (PSD) of MCC bulk solution and supernatant.

5.4 Solubility Testing of Stored Samples

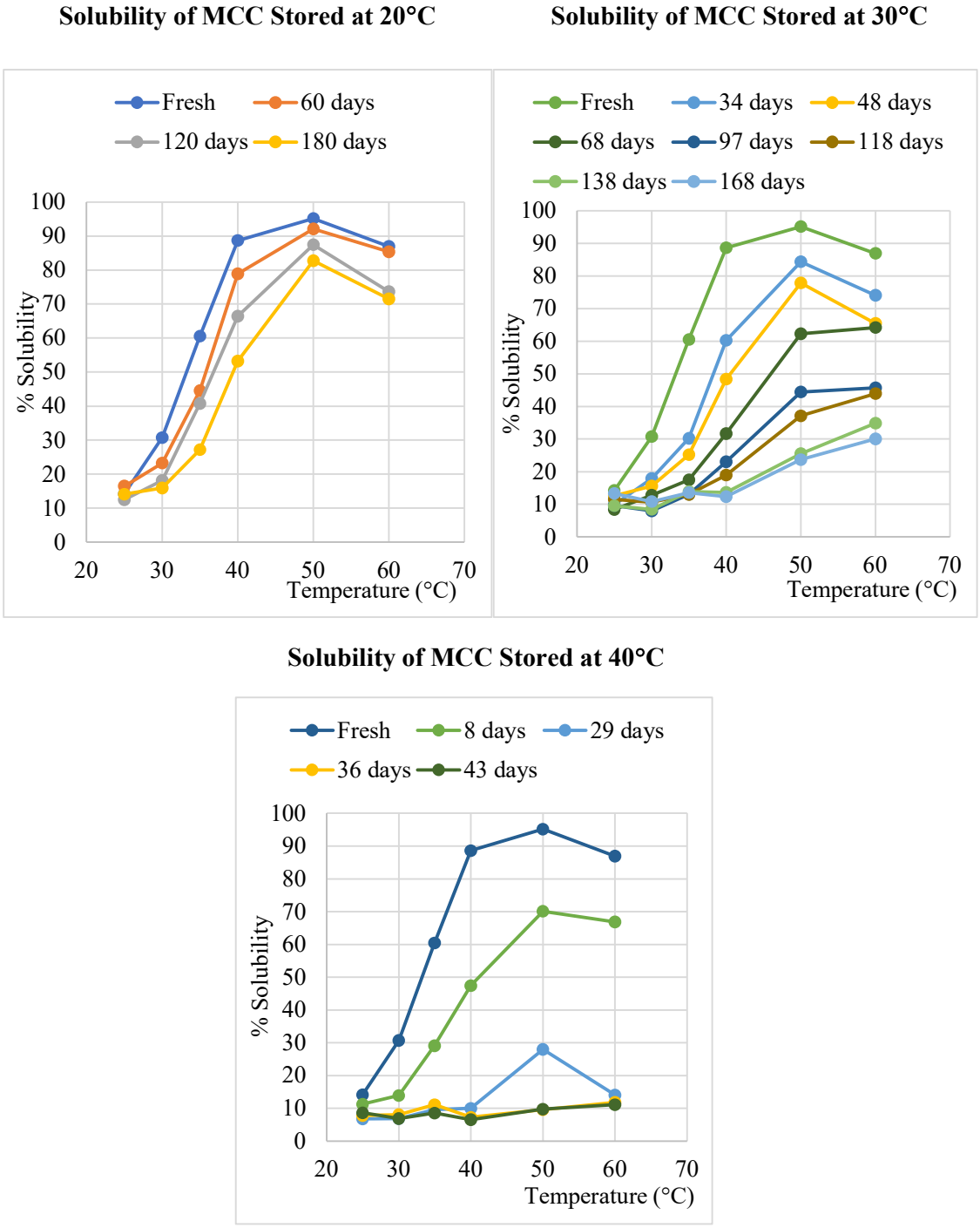


Figure 5.3 Soluble reconstituted MCC solution as a function of hydration temperature stored at different temperatures. Note: Error bars are not indicated as the uncertainties are much smaller than the data symbols in the graph.

The solubility tests were performed on stored MCC samples of different temperatures such as 20°C, 30°C and 40°C and the individual results of storage temperature can be viewed in the Figure 5.3. From the results of fresh solubility MCC reconstituted solutions, the study found hydration temperature had stronger influence. But in the case of hydration time, even at 30 min, the maximum solubility can be attained. So, the effect of storage time and temperature on the solubility of MCC solution was tested under different hydration temperature of 25, 30, 35, 40, 50, 60°C for the hydration time of 30 min.

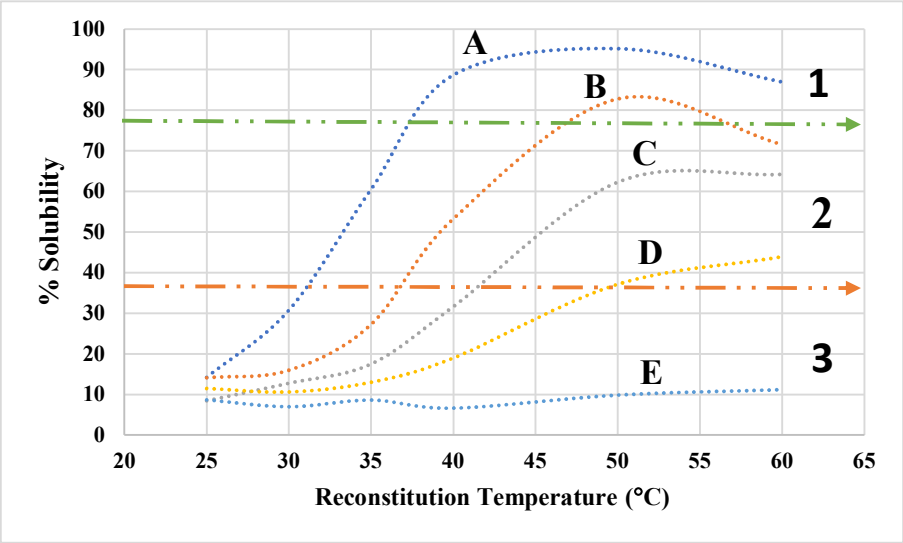


Figure 5.4 General Solubility Curve Pattern of Fresh and Stored MCC Samples.

The results of fresh and stored samples seem to follow a similar general trend but in stored samples, there is a decline in solubility with respect to storage temperature. These curves can be sectioned into three regions (see Figure 5.4) to discuss the impact of storage temperature on soluble material of MCC with the hydration temperature of 50°C. The curve (A) denotes fresh MCC powder falls under region 1and the curve (B) 20°C stored MCC samples falls under region 1&2. The curve (C) denotes 30°C stored MCC samples falls under region 2 and then followed by curve (D) which indicates 30°C stored MCC samples falls under region 2&3. Finally, the curve (E) denotes 40°C Stored MCC samples falls under region 3.

In Region 1, the initial change happening with respect to storage temperature and time and results in slight decrease in numerical solubility percentage for any given hydration temperature. The decrease in the solubility from 95% to 80% of A and B clearly denotes the initial changes occurring with respect to storage conditions. Region 2 can be described as the period where maximum changes happening owing to storage temperature and time for any

given temperature. The solubility curve pattern of C and D falls under region 2 and maximum changes can be seen through the decrease in the solubility from 95% to 40%. In region 3, MCC solubility is largely lost with a minimum solubility of about 10% that can be found in the solubility curve pattern of E which is likely due to the lactose, soluble minerals and residual whey proteins.

In 20°C stored MCC sample, the solubility initially remained in region 1, but gradually based on storage days it was declining and fallen in the region 2 when it is about 4 and 6 months. For the 30°C stored MCC powder, when it was about 1-month time, solubility initially fallen in the region 1 and decreased to region 2 rapidly with storage time. The gradual decline in the soluble nature of the powder was observed throughout the time and complete solubility dropped for 6 months. MCC stored at 40°C showed no region 1 and immediately moved to region 2. After 8 days, the curve reaches minimum solubility and the curve reaches a plateau of 9% soluble material in a month period.

5.5 Comparison between Storage Temperature

The different range of storage temperatures were selected to reflect the conditions that the product could be exposed commercially. The 40°C stored samples considered to be extreme temperature studied in comparison to the room temperature 20°C stored samples. The earlier section described the impact of storage temperature and discussion was based on the solubility curve pattern. In this section, Figure 5.5 illustrates the soluble material percentage of MCC powders stored at different temperature as a function of storage time.

The Figure 5.3 explains storage temperature impact and caused marked solubility reduction for any given hydration temperature. From the result of Figure 5.1, a study found the best hydration temperature of MCC is 50°C for the maximum solubility. So, the soluble material percentage at 50°C hydration temperature was plotted against the function of storage time in the Figure 5.5. The graph explains the higher the storage temperature, the faster solubility decline rate in MCC samples. The rate of solubility decrease on storage (denoted by the slope value) increases significantly with increasing storage temperatures. The MCC solubility curve for 40°C (-2.26 % solubility/day) displays a rapid decline and in a storage time of 30 days complete soluble material lost in the samples. The solubility curve of 30°C MCC samples (-0.48 % solubility/day) gradually decrease in solubility and when after 180 days, the solubility was completely lost. The 20°C stored samples (-0.073 % solubility/day) had a linear decrease in

solubility from 99% to 80% over a storage period of 180 days and these results were similar to Nasser et al. (2017).

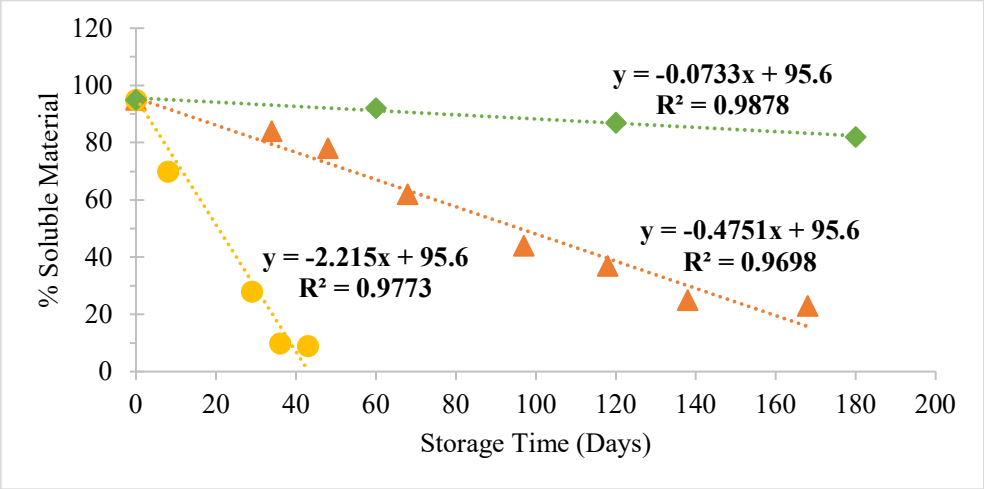


Figure 5.5 Solubility data (reconstitution temperature 50°C for 30min) as a function of storage time for MCC stored at temperatures of (◊) 20°C, (Δ) 30°C, (O) 40°C.

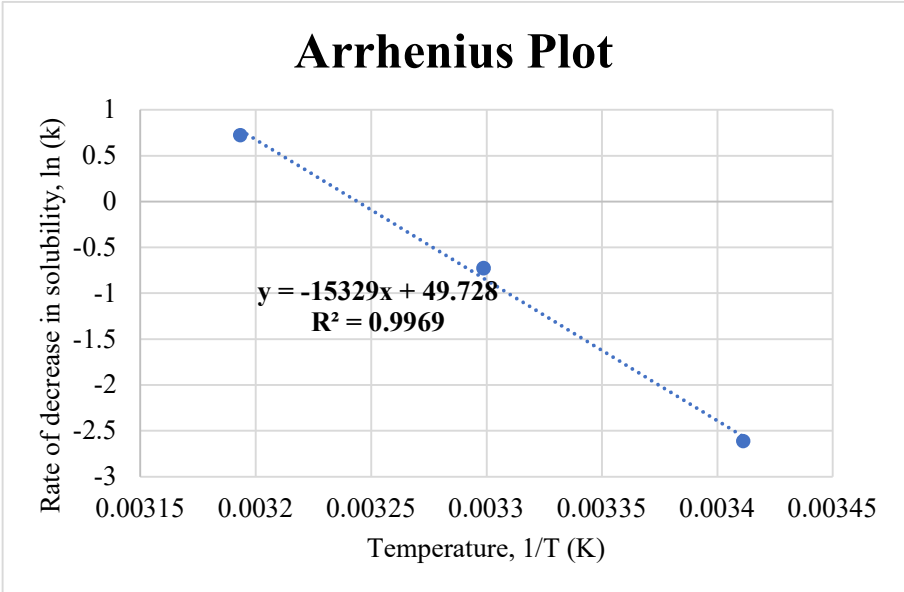


Figure 5.6 Arrhenius Plot of the Different Temperature Stored MCC Samples.

Figure 5.5 displays the relationship between the solubility (50°C/30min) of MCC and the age of the powder when stored over a range of temperatures (20, 30 and 40°C). The solubility decreases with storage time, at each temperature, can be adequately described by a linear model with correlation coefficients >0.96. The solubility loss of MCC powder is a dynamic process whose temperature sensitivity follows Arrhenius equation as shown in Figure 5.6. The slope of the Arrhenius plot indicates that the rate of solubility loss increases by a factor of 5.3x for every 10°C increase.

5.6 Discussion

The following discussion is some of the reasons for the prolonged rehydration and insoluble nature of MCC. Among the powder components, the insoluble material is partly to be related to adhesion of fat globules with casein micelles (McKenna et al., 1999). But, the presence of fat content in the commercial powder used in this study is 3.0% which is a substantial amount to be considered as a significant factor for the insoluble part of MCC. Furthermore, the minimal amount of lactose presence found had no impact on the MCC powder dispersion as there were no interactions between lactose and micelles (Gianfrancesco, Casteran, Andrieux, Giardiello, & Vuataz, 2011).

Excluding fat and lactose, another major component present in the MCC powder is caseins, which might be the reason for the cause of insolubility. The authors (Anema et al., 2006; Fang, Rogers, Selomulya, & Chen, 2012) found interactions between caseins as the cause for insoluble material in the MPC powder. Further, study by these authors (Gazi & Huppertz, 2015; Havea, 2006; McKenna et al., 1999) proposed that the issues associated with high-protein casein powders are the slow release of components prolong the rehydration mechanism rather than the actual insoluble material formation.

In Gazi and Huppertz (2015) study on MPC85 which tested the effect of reconstitution time and temperature on the solubility of fresh powders. A study resulted in strong increasing solubility with reconstitution time and particularly with increasing reconstitution temperature. The similar result was obtained for MCC powder in this study when considering reconstitution time and temperature for rehydration.

The possible reasons for the decrease in solubility at hydration temperature higher than 50°C could be milk protein denaturation and aggregation with respect to the increased hydration temperature. Qian et al. (2017) study found that whey protein denaturation degree and association of whey protein with casein increased as the hydration time and temperature increased. Vasbinder and De Kruif (2003) explained the whey protein denaturation occurs in two forms; 1) Soluble protein aggregates 2) Associate on the surface of the casein micelle aggregation. Patel, Singh, Anema, and Creamer (2006) postulated that the prolonged heating time could promote the protein's hydrophobic group interaction, which could further facilitate the combination of κ-casein and β-lactoglobulin. However, protein denaturation increases with heating temperature above 60°C, but in our study, solubility declined after the hydration temperature of 50°C. In contrast, Amelia et al. (2013) study reconstituted MCC at the

temperature of 65°C for the production of low-fat cheddar cheese. From the result of this study, for the stronger gelation and bond strength, reconstitution temperature should be considered for the complete solubilisation of MCC. Further study needs to be conducted to find out what kind of protein reaction could have caused the solubility drop.

A possible mechanism that might explain insolubility is cross-linked protein network formation over the MCC powder particle surface that could act as a barrier for water absorption and result in longer hydration time. The cross-linking degree would be expected to increase due to storage conditions. The recent study Nasser et al. (2018) found MCC powders possessed a considerable amount of high-molecular-weight (HMW) complexes prior to storage and these complexes produced through the technological powder manufacture steps. Anema et al. (2006) reported the degree of cross-linking increases as the storage time and temperature increase which can be analysed by electron microscopic techniques.

While, lactose is present in a minimal amount it has been implicated in loss of solubility in high protein powders. Previously published articles found lactose initiates Maillard reactions owing to storage temperature. The causes for the decline in solubility and a solution to improve rehydration condition of MCC sample has been studied in the following chapter.

5.7 Summary

In this chapter, the influence of hydration time and temperature on the solubility of fresh MCC powder was studied. It was found that both factors strongly influenced powder solubility. The rehydration time focussed on in this study will benefit industrially by reducing the mixing time. The solubility declined at reconstitution temperatures above 50°C in both the fresh and stored samples. Further research in this area would be of great help for better understanding of protein reactions that happens in MCC.

The study also focused on the effect of storage time and storage temperature on the solubility of MCC powder and found the solubility decline rate is higher as the storage temperature and time increases for any given hydration temperature. When the storage temperature is raised above 20°C, the solubility decline rate is higher. It was observed that 40°C stored powder lost solubility in a storage time of one month. This gives a picture about how the storage temperature above 40°C, which the powder may be exposed to during shipping through hot climates may influence the powder's solubility characteristics.

6. Solubility Deterioration of Stored MCC

6.1 Introduction

The previous chapter showed that the solubility characteristics of the MCC powder is significantly influenced by the storage conditions such as temperature and time. The same result has been shown for MPC powder which has been studied extensively with a detailed emphasis on the gradual solubility loss owing to the storage conditions (Anema et al., 2006; Gazi & Huppertz, 2015; Mimouni et al., 2010a). Currently, the most commonly cited reasons for the lack of rehydration properties are 1) Maillard reactions in the presence of lactose and 2) Protein cross-linking at the surface of the powder or by the close micelle association.

High-casein protein content powders such as MPC and MCC are suspected to have a similar rehydration phenomenon, but less research has been done on MCC with the focus on studying the reasons behind the cause of insolubility. It also appears from the literature that the colorimetric change in ageing MC powder such as browning index has not been reported well enough for MCC powder. To visualise the potential protein cross-linking, as has been observed for MPC, the microstructure of the fresh and stored dairy powders was analysed through combined electron microscopic analysis of scanning and transmission. As SEM shows the picture of powder particle surface and TEM shows the picture of the cross-sectional view of the powder.

Studying the cause of the insolubility is very important because it leads to understanding the nature of the powder. However, when a solubility loss occurs in a commercially manufactured powder as we could see in Chapter 5 it is important to find an alternative industrially available option for rapid dissolution of the dairy powders to improve complete solubilisation regardless of the storage conditions. As dissolution is considered to be a rate-limiting step in casein-based powder rehydration, some attempts have been made for MPC powders with increased solubility using high shear mixing. By generating shear-force a high-pressure homogenizer, which is traditionally used for disrupting fat globules, can be utilised for particle size reduction and thereby accelerate powder rehydration. This chapter aims to study the common two causes for the decrease in solubility during storage i.e., analysing browning index to understand Maillard reactions in the presence of lactose and to observe structural impact of protein cross-linking through electron microscopic analysis. The chapter is also focussed on solutions to improve the solubility of the stored samples to overcome the potential for customer complaints that would be faced in the industries.

6.2 Results

6.3 Possible Reasons behind Solubility Decline Rate in Stored Samples

6.3.1 Colour Analysis

Using the methodology explained in the Section 4.3.3, the colour analysis of fresh and stored MCC samples were measured and the colour coordinates (L*, a* and b*) were utilised to derive the browning index (BI) parameter. These MCC sample findings stored at different temperature of 20°C, 30°C and 40°C has been plotted against storage time in the Figure 6.1.

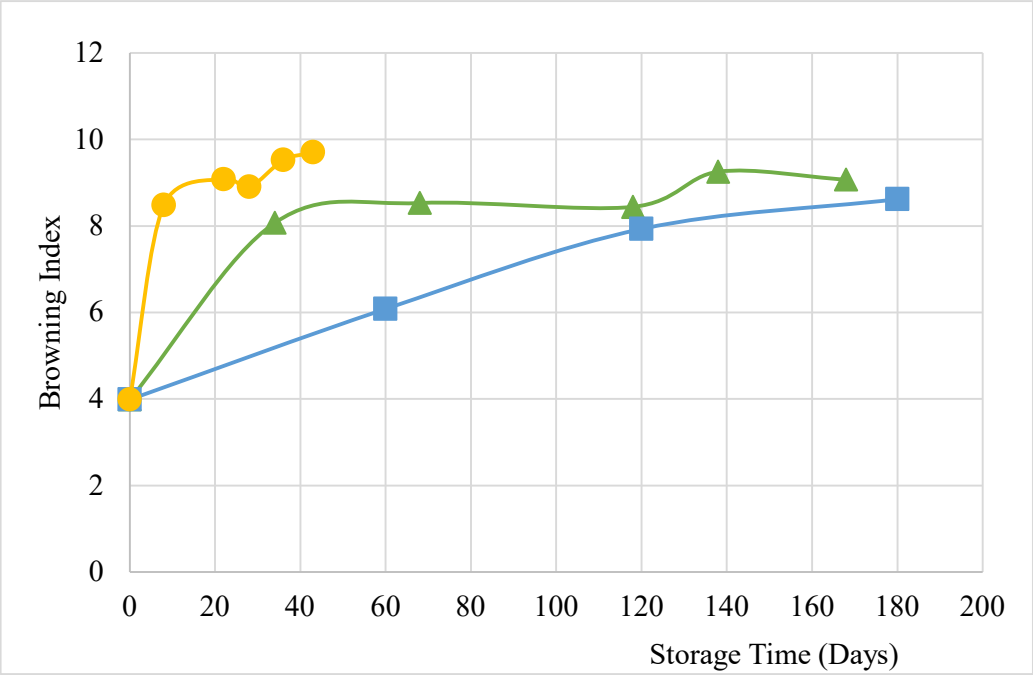


Figure 6.1 Browning index as a function of storage days for the MCC samples stored at temperatures of (■) 20°C,(▲) 30°C and (●) 40°C.

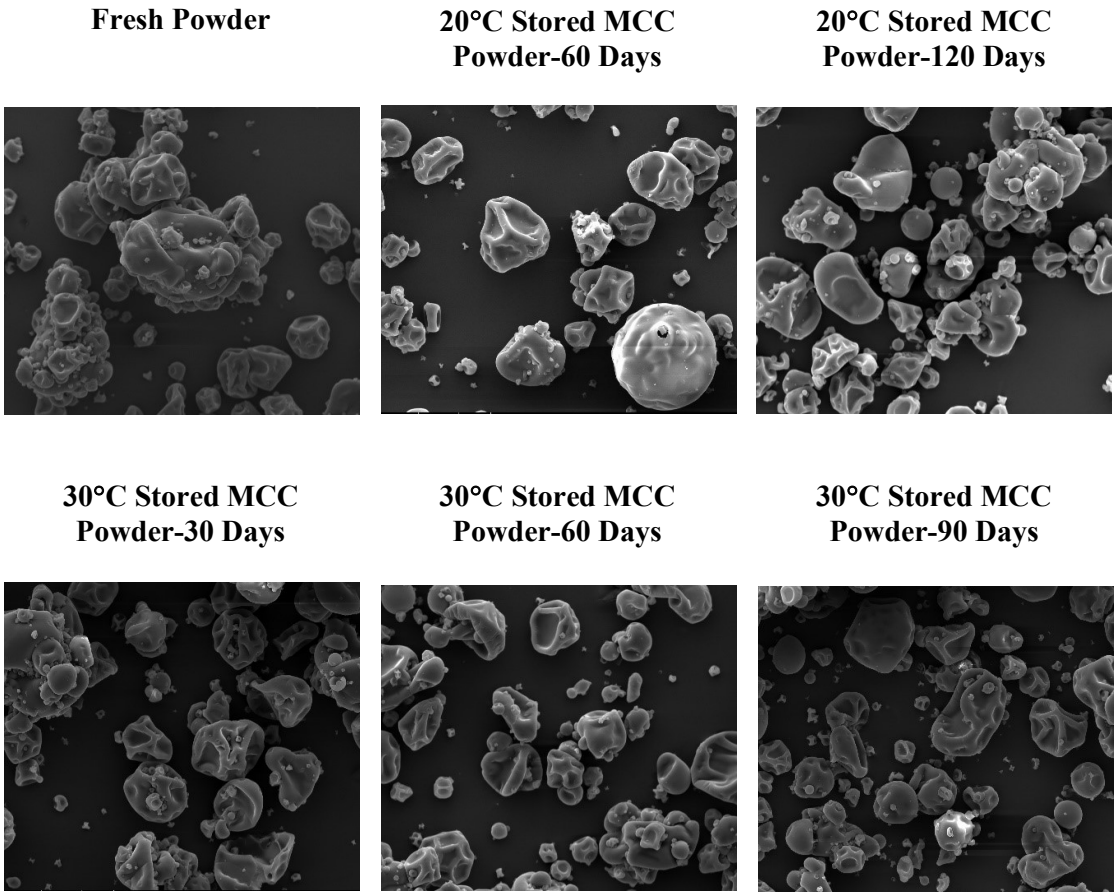
The already published research work on MPC samples observed storage temperature strongly influenced browning index. As expected, we could observe the same phenomenon for MCC samples from Figure 6.1. And also, the faster browning rate occurred for the samples stored at 40°C indicates that with higher storage temperature, faster browning rates occur. However, the increase in brown coloration is not detectable by the naked eye but was able to be derived by measuring colour coordinates. For the samples stored at 20°C in comparison to reference powder, the BI slightly increased from 4 to 6 in a 2-month period and continues the same trend for the total 6-month period. At 40°C, a BI of 8.5 was achieved after only 8 days of storage

which at 20°C took 180 days of storage. So, storage time and temperature were shown to have an effect on the Maillard reaction.

6.3.2 Microstructural Analysis

6.3.2.1 Scanning Electron Microscopy

The microstructure of MCC powder particles has been analysed using SEM to study particle size, shape and also to get insights of microscopic features such as pores or wrinkles. The SEM images of fresh and stored MCC powder particles of 500x magnifications can be viewed in Figure 6.2. Based on different magnifications, the spray-dried average particle diameter varied from 50-500 μm indicates these particles are casein micelles. Generally, the shape of the particles appears to be spherical. The particles have a smooth surface but with a high degree of shrinkage as demonstrated in earlier studies of Tamime, Robinson, and Michel (2007) which was attributed to the protein material's strong compaction and shrinkage especially casein micelle during spray drying.



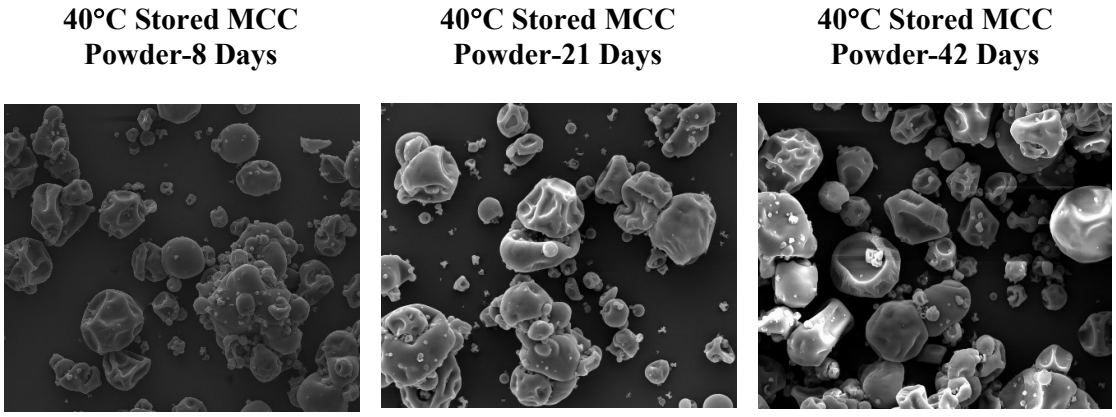


Figure 6.2 SEM microstructural view of fresh and stored MCC powders.

Differences between the microstructure of fresh and stored MCC powder particles was not evident. Furthermore, there are no visible changes between different temperature stored MCC powder particles. Even Mimouni et al. (2010a) study reported there are no visible changes between powder particles however, a significant difference has been analysed when the powder particles are rehydrated. Future studies need to be conducted to find out the microstructural difference between stored and fresh powder particles.

6.3.2.2 Transmission Electron Microscopy

The microstructure of MCC powder particles has been analysed using TEM to study particle size and cross-sectional view which gives insights about protein-protein interaction such as micellar aggregation. The TEM images of fresh and stored MCC powder particles of 20,500x magnifications can be viewed in Figure 6.3. From all these micrographs, the most distinctive features of the fresh and stored MCC powders represent closely-packed casein micelles and micelles interaction. In these powders, there was no significant difference in the casein micelle structure and in their interaction. There were no earlier studies viewed MCC powder's microstructure using TEM. McKenna et al. (1999) studied whole-milk powder and observed fat globules associated micelles and denatured whey protein-κ-casein complex. However, the fat content in MCC powders is very low, these images only show tightly-packed casein micelle. There is no visible difference between the fresh and stored MCC powder regardless of storage temperatures. In future, the method development of TEM is required to find out the possible reasons for micellar aggregation such as extra skin layer formation, if it occurs around casein micelles of MCC powder.

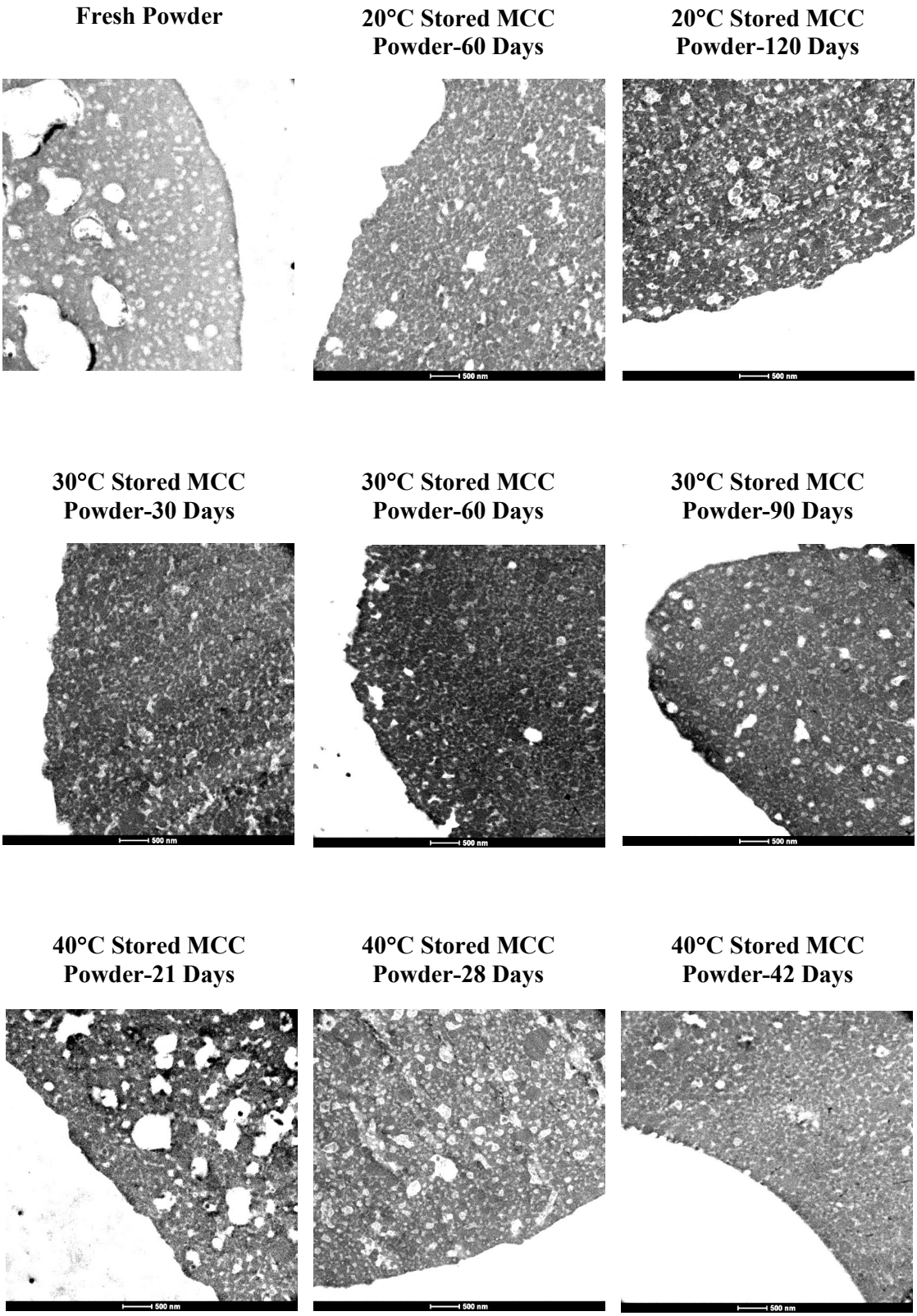


Figure 6.3 TEM Cross-Sectional View of Fresh and Stored MCC Powders.

6.4 Improving the Solubility of Stored Samples

The impact of increasing shear force generated using a single-stage high pressure homogeniser with three different pressures (100, 150 & 200 bar) on solubility was examined. Using the methodology explained under Section 4.3.1 and 4.3.2, the solubility determined by centrifugation are shown in Figure 6.4. To further investigate the solubilization of the powder, the PSD analysis has been done and results can be viewed in the Figure 6.5.

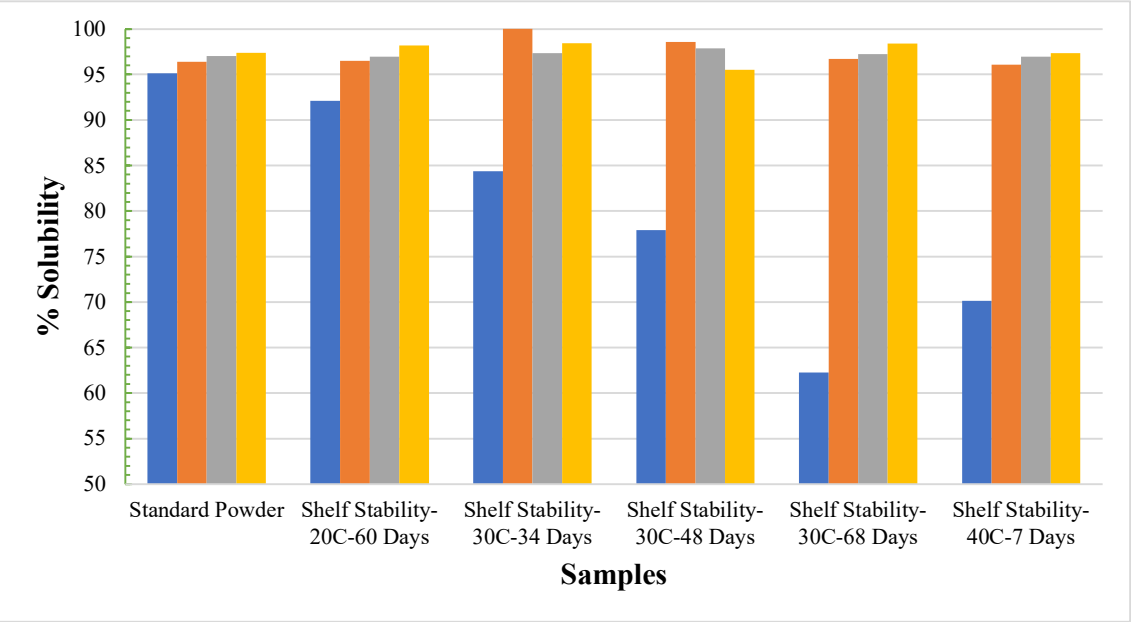
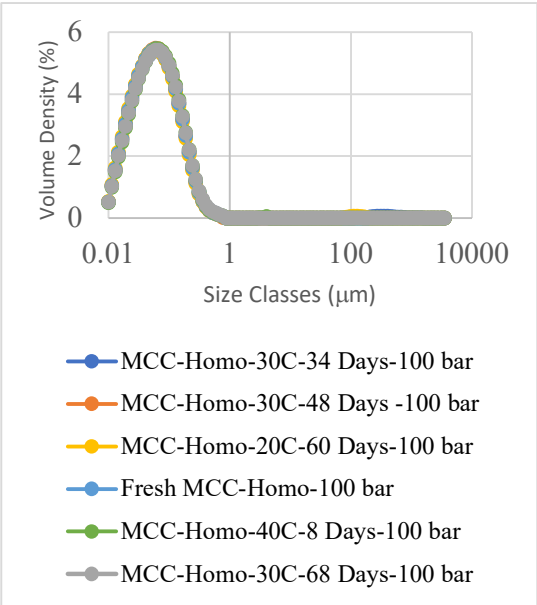


Figure 6.4 Solubility (reconstitution temperature 50°C) of fresh and stored MCC samples before (■) and after homogenisation at three different pressures of 100 bar (■), 150 bar (■) and 200 bar (■). Note: Error bars are not indicated as the uncertainties much smaller than the data values in the graph.

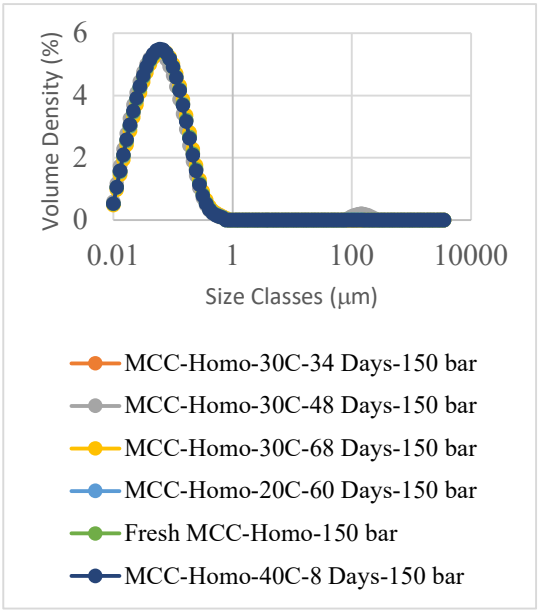
The blue clustered column indicates solubility before homogenisation, several powders stored under a range of conditions were examined. The solubility of these powders before homogenisation ranged from 63 to 95 %. Regardless of pressure change, the solubility increased for stored samples from 95 to 100 %. The PSD for fresh and stored MCC samples are broadly consistent with the solubility data values without the appearance of bimodal distributions. However, the premixing step performed at 50°C for 30 min and then followed by homogenisation. From the combined solubility analysis, it is recommended to use homogenisation with low pressure setting to overcome the industrial issue of using stored samples. The application of single-stage high pressure homogeniser is an effective approach to

breakdown the powder agglomerates and promote the release of individual casein micelles into the solution.

A. PSD Analysis of Homogenised MCC Sample at 100 Bar



B. PSD Analysis of Homogenised MCC Sample at 150 Bar



C. PSD Analysis of Homogenised MCC Sample at 200 Bar

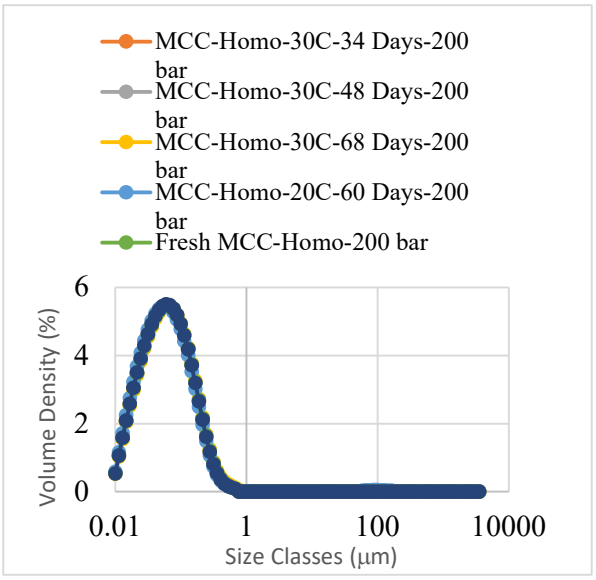


Figure 6.5 Particle size distribution (PSD) of Homogenised MCC Sample at different pressures of 100 bar (A), 150 bar (B) and 200 bar(C).

6.5 Discussion

The present findings on the basis of the browning data indicates that progressive insolubility may be correlated with Maillard browning and thus the presence of lactose. This result was consistent with Anema et al. (2006), who reported using mass spectrometric analysis found casein in MPC85 became lactosylated. In the Maillard reaction, the initial step is the interaction of ε-amino group of lysine and lactose results in protein lactolysation reaction. It is found to be associated with protein crosslinking reaction products such as lysinoalanine and histidinoalanine. This is essential to note that Maillard reactions might not directly lead to protein cross-linking but instead, they stimulate changes in protein properties which also favours cross-linking. This phenomenon has been proved by Nasser et al. (2018) in which lactose-free and standard MCC has been studied. The conditions which favour Maillard reactions also favour protein crosslinking, which altogether results in insolubility. Hence, a measure of browning index can be a factor to predict cross-linked products. It is also an indicator of Maillard reaction taking place which could indicate the reason for the decline in solubility rate of MCC.

Among the two most commonly cited reasons, Maillard reactions taking place and hence, proved by the colour analysis and it is also said to be the indirect cause of protein crosslinking. This theory was studied through microstructural scanning and transmission electron microscopic analysis for fresh and stored MCC samples. The skin formation observed by McKenna et al. (1999) for MPC on the surface of the powder particles was not found on the MCC, as the study performed on the dry state rather than rehydrated form. The same result was mentioned by Mimouni et al. (2010a) and Burgain et al. (2016). Furthermore, elucidated gold coating over stub in SEM pre-sample preparation step is thicker than 5 nm, that may have overlay variations in the fresh and stored powder's surface profile. As there are no earlier

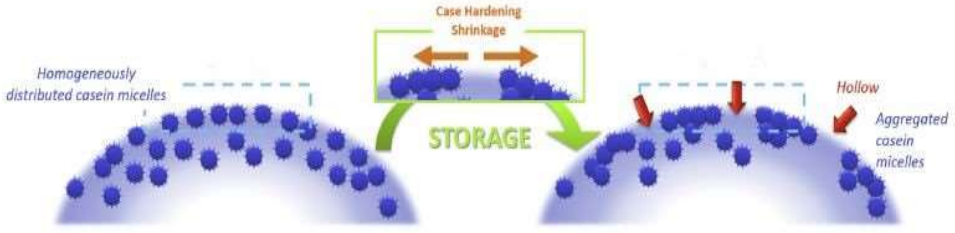


Figure 6.6 Possible Mechanism Happens at Particle Surface During Storage (Burgain, Scher, Petit, Francius, & Gaiani, 2016).

studies used TEM for MCC samples and the main composition in MCC is of casein micelle, further method development should be developed and studied.

Casein micelles are spatially separated in fresh powders thus preventing the cross-linking between caseins. After high-temperature storage, casein micelle aggregation leads to a heterogeneous surface where a monolayer skin of tightly packed casein micelles form and that minimise water uptake rate upon reconstitution (Mimouni et al., 2010a). The underlying mechanisms proposed are protein aggregation through covalent (intermolecular disulphide bonds) and non-covalent (hydrophobic) interactions that end-up resulting in surface hardening and insolubility as shown in Figure 6.6 (Burgain et al., 2016).

The finding of the present study also suggests when there is a solubility drop in the stored samples, a high shear force generated by single-stage high-pressure homogenisation was the effective solution to break apart the powder agglomerates and release the single particles into the solution. The result is in line with the literature (Chandrapala et al., 2014) that found using a homogeniser was a powerful approach yet the study showed solubility as 84% for MCC and also a bimodal distribution in PSD analysis at 80 bar. In contrast, the solubility attained in this study was above 95% for all stored samples without bimodal distribution in PSD analysis regardless of pressure change. However, the dissolution of powder particles to a certain extent has been done through pre-treatment stirring step at 50°C. Subsequently, the pre-dissolution followed by homogenisation for stored samples is found to be a more efficient step for the complete solubilisation of the MCC powder particle.

6.6 Summary

This chapter examined the causes of the solubility drop in stored MCC samples in comparison to the fresh ones. Results of the browning index showed that even low content of lactose initiates Maillard reaction in the stored samples. Though the protein crosslinking was not visible in the stored MCC samples using the microstructural SEM and TEM analysis. It gave an additional insight of particle size, shape and cross-sectional view such as protein-protein interaction of casein micelles in MCC powder particle. A single-stage high-pressure homogeniser can be an efficient approach to overcome the solubility decline in the stored samples. This finding helps to produce nutritional food products by thoroughly dissolving the dairy ingredient and also can be recommended as the solution for the exported MCC powders in the industrial setting.

7. Rennet Gelation Characteristics of Skim Milk-Fortified Fresh MCC Powder

7.1 Introduction

For increasing the cheese yield, milk concentration is usually carried out by ultrafiltration (UF) in the dairy industries. Concentrating milk with microfiltration (MF) is theoretically appropriate for cheese production compared to UF milk as it enables whey protein removal before cheese production. As a protein source for cheese manufacture, MCC obtained by MF might be a good alternative ingredient for protein fortification of the cheese milk base. The preparation of protein-fortified cheese milk base results in the modifications of chemical composition, which has been shown to influence the rheological and physical properties of cheese (Lucey, Johnson, & Horne, 2003).

The reconstitution of dry ingredients into a solution is necessary for many food products. The reconstitution process requires a period of time before further processing can take place. In several cases, this hydration time can be helpful to evaluate the properties of the desired product, in this case, predominately the gel structure. It would be advantageous for industry to be able to reduce the hydration time. The milk coagulation characteristics in this study are determined by the parameters gelation time, gel strength, gel firming rate and optimal cutting time using the Formagraph and the low-amplitude oscillation rheometry (LAOR). These parameters play a significant role in optimising the production of large-scale fermented dairy products.

This chapter aims to investigate the effect of skim milk/MCC fortification on the cheese properties. In addition, the renneted skim milk/MCC solutions with two different hydration times of 24 hr and 3 hr were prepared to quantitatively measure the effect of the hydration time on the rennet gelation properties of the solutions. Both the dynamic gel firmness tests (LAOR and Formagraph) were used in this study to derive industrially relevant parameters. However, LAOR was able to measure only one sample at a time while the Formagraph gives the advantage of measuring 10 samples at a time. This study also compared both the techniques for reliable results on rennet gelation characteristics.

7.2 Results

The samples of fresh powder were prepared as two replicates according to the protocol described in Section 4.2.3 and 4.2.4. The longer hydration time samples are termed as 24 hr MCC replicate 1 & 2 and the shorter hydration time are termed as 3 hr MCC replicate 1 & 2.

The two experimental methods were used to study the rennet gelation characteristics of MCC as described in Section 4.3.7 and 4.3.8.

7.3 Effect of Hydration Time on the Rennet Gelation Property of Skim Milk/MCC Solutions

The solubility, PSD and pH analysis were analysed for the prepared samples of two different hydration times (24 hr and 3 hr) as explained in the Section 4.3.1 and 4.3.2 and the results are shown in Figure 7.1 and Table 7.1.

Figure 7.1 Skim Milk-Fortified MCC Solution's PSD Analysis of 24 hr and 3 hr Hydration Time.

<table>
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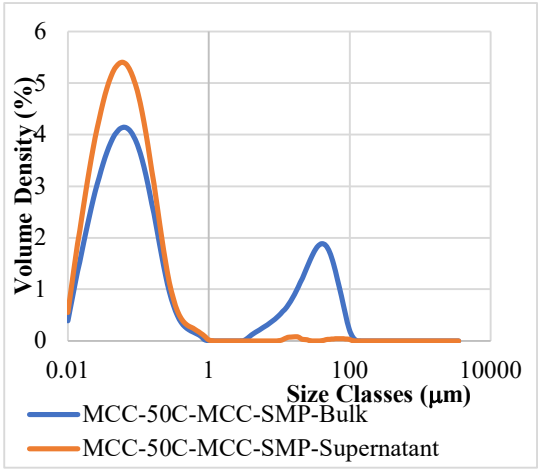
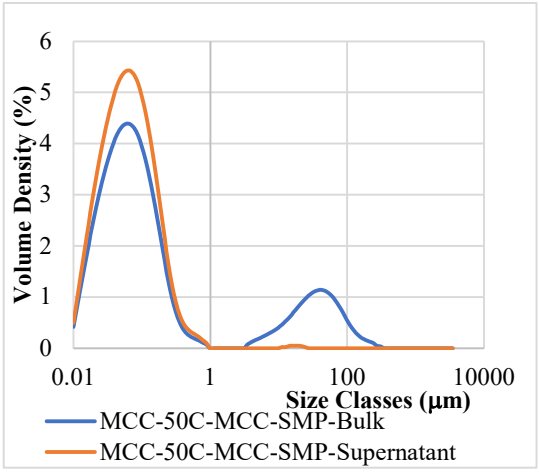
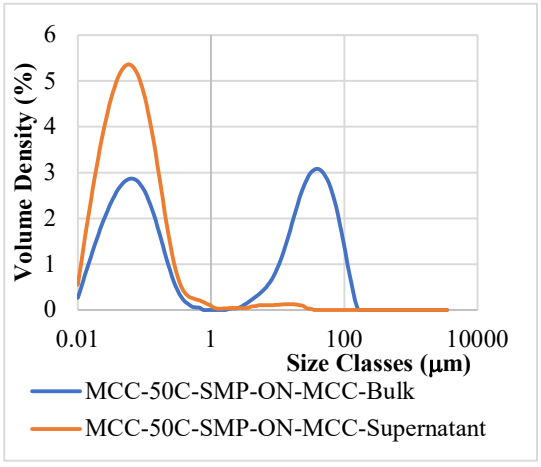
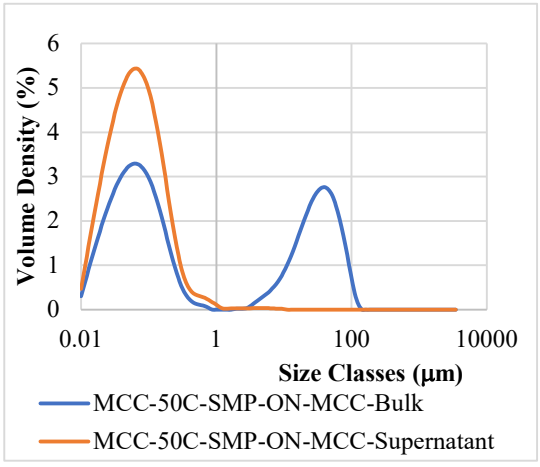
<tr><td> 
</td><td> 
</td></tr>
<tr><th>Solubility – PSD Analysis – 3 hr-MCC-Replicate 1</th><th>Solubility – PSD Analysis – 3 hr-MCC-Replicate 2</th></tr>
<tr><td> 
</td><td> 
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Table 7.1 Percentage Solubility and pH of the Measured Samples.

	24 hr-MCC-Replicate 1	24 hr-MCC-Replicate 2	3 hr-MCC-Replicate 1	3 hr-MCC-Replicate 2
% Solubility	99.2834	99.0875	98.3826	98.8753
pH	6.67	6.71	6.67	6.70

7.3.1 Low-Amplitude Oscillation Rheometric Analysis

Table 7.2 provides the milk coagulation characteristic parameters derived using LAOR analysis for 24 hr and 3 hr hydration time. The coagulation temperature of 30°C with sample pH of 6.7 was used as the experimental condition. The RCT has been defined as when G' is above a fixed value (0.2 Pa) and also can be defined as when there is a consistent increase in G' (Fox et al., 2017). When comparing the longest and shortest hydration time, the shortest (3 hr) resulted in shorter gelation time and higher gel strength. But the resulting values had a variation for the same hydration time.

Table 7.2 Milk Coagulation Characteristics of Renneted Skim Milk/MCC Solution Using LAOR Analysis.

Sample Name	Gel Time, RCT (min)	Maximum Gel Firmness (Pa)		Gel Firming Rate (Pa/min)	Set-to-cut (SCT) at 20 Pa (min)
		After 30 min	After 180 min		
24 hr MCC-Replicate 1	13	69.9	504	0.0502	21
24 hr MCC-Replicate 2	14	52.5	490.5	0.0497	23
3 hr MCC-Replicate 1	11	112.3	619.7	0.0592	18
3 hr MCC-Replicate 1	12	93.8	527.1	0.0507	18.5

7.3.2 Formagraph Analysis

Table 7.3 provides the milk coagulation characteristics parameters derived using Formagraph analysis for 24 hr and 3 hr hydration time. The coagulation temperature of 30°C with sample pH of 6.7 was used as the experimental condition. All the parameters are retrieved by

processing the saved *.fmg* file in the digital Formagraph software. As in LAOR analysis, when comparing the longest and shortest hydration time, the shortest (3 hr) resulted in shorter gelation time and higher gel strength. The replicates had a similar resulting value for all the parameters.

Table 7.3 Milk Coagulation Characteristics of Renneted Skim Milk/MCC Solution Using Formagraph Analysis.

Sample Name	Gel Time, RCT (min)	Maximum Gel Firmness, a30 (mm)	Gel Firming Rate (mm/min)	Optimal Cutting Time, k20 (min)
24 hr MCC-Replicate 1	22	24	0.035	28
24 hr MCC-Replicate 2	22	25.3	0.035	28
3 hr MCC-Replicate 1	19	37.3	0.042	24
3 hr MCC-Replicate 2	19	40.2	0.043	24

7.4 Comparison between LAOR and Formagraph

By comparing both methods, LAOR and Formagraph showed good agreement between the parameter trends measured which is consistent with the findings by (Auldist, Mullins, O'Brien, & Guinee, 2001; Ketto, Schüller, Rukke, & Grethe, 2015). However, the parameters cannot be compared directly as there is a variation in the measured values. The Formagraph showed a longer gelation time on average compared to LAOR. The possible reason for a shorter gelation time in rheometry is due to higher sensitivity and also non-destructive measurement type (Ketto et al., 2015). Furthermore, Frederiksen et al. (2011) mentioned that the Formagraph generates analog form of chart output as a disadvantage. But in the current digitalised world, the software makes it easier to convert analog chart form into the numerical format.

Though the values are not same, both the methods showed 3 hr hydration time of MCC had faster gelation and optimal cutting time, further higher gel strength and firmness rate in comparison to 24 hr hydration time of MCC. The results can be viewed in Figure 7.2 and 7.3. The findings show that Formagraph can be used as an alternative approach for large samples

of milk coagulation study. A key advantage of the Formagraph is it can generate results for 10 samples in a shorter time of 30-60 minute. The fast rate of data collection from the Formagraph was particularly desirable when time for experimentation was curtailed due to COVID-19 lockdowns.

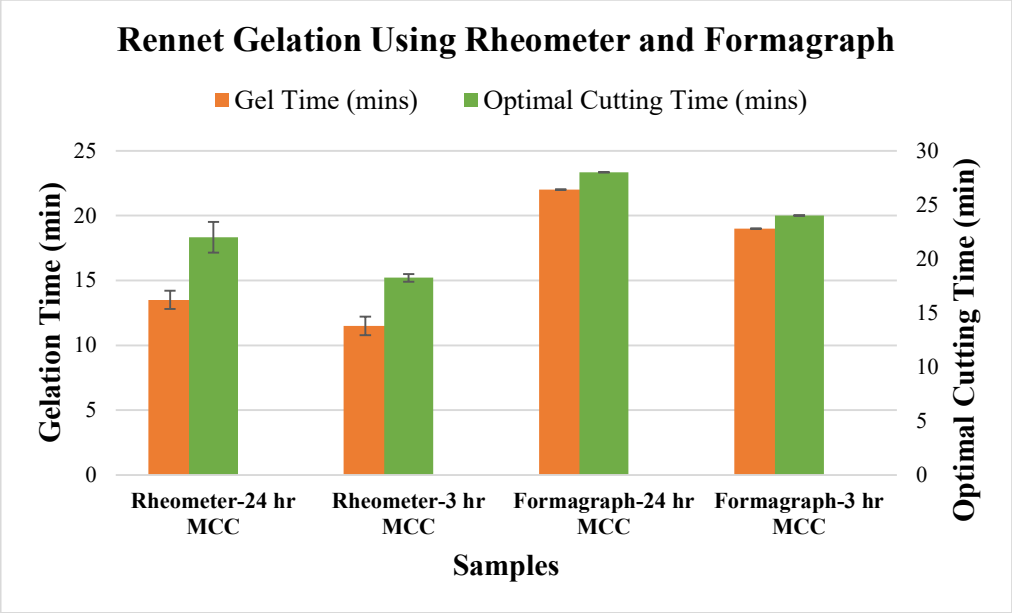


Figure 7.2 Mean and Standard Deviation (SD) for the gelation time and optimal cutting time of 24 hr and 3hr hydration time MCC solution using LAOR and Formagraph analysis.

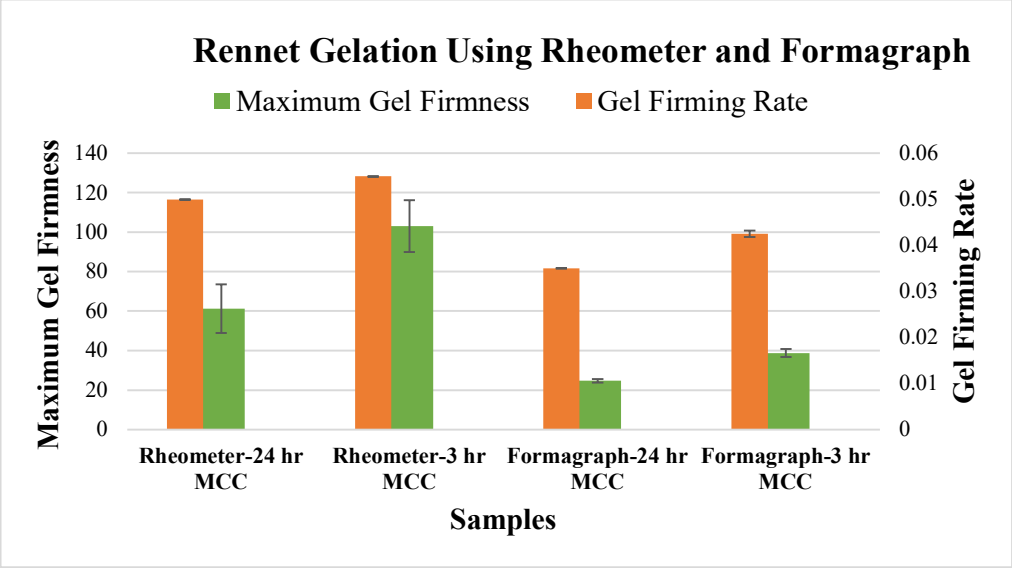


Figure 7.3 Mean and Standard Deviation (SD) for the MGF and GFR of 24 hr and 3hr hydration time MCC solution using LAOR and Formagraph analysis.

7.5 Discussion

As already mentioned for the effective expression of powder's gelation characteristics, the product should be completely dissolved in a solution. To evaluate this, the solubility of skim milk-fortified MCC was analysed and found to be 99% which was similar to the solubility of MCC-dissolved in water but these results were contradictory to Hunter, Hemar, Pinder, and Anema (2011). And also, the study mentioned MPC gelation time has 30 min, which was higher in comparison to the gelation time of 20 min for MCC.

Furthermore, Lin, Kelly, O'Mahony, and Guinee (2016) mentioned MCC fortified solution resulted shorter gelation time with higher gel strength in comparison to other casein ingredients. It also empathised the secondary aggregation phase of gelation is strongly influenced by the mineral equilibrium. This phenomenon was similar to the most of the previous rheological studies (Ferrer, Hill, & Corredig, 2008; Sandra & Corredig, 2013) that showed higher insoluble calcium levels results in higher gel strength. Martin, Williams, and Dunstan (2010) reported the insoluble calcium was affected during the manufacturing process of MCC mainly in the diafiltration stage. As this process increases protein concentration by further removing minerals and lactose and thereby modifies ionic equilibrium. The similar result was analysed by the recent Zhao et al. (2020) study which also reported κ-casein hydrolysis and casein micelle aggregation were inhibited in the diafiltered casein micelle suspensions. However, the study found a similar soluble phase composition was able to be re-established when the concentrates were recombined with the skim milk.

The reconstitution of MCC powder into the skim milk base has a significant impact on the rheological properties of the cheese. As the hydration time increases, the onset of gelation and optimal cutting time also increases when observed through both Formagraph and rheometric analysis. Increase in hydration time increases the soluble casein, but the rennet concentration remains constant. The extended gelation time also might be due to the protein/rennet ratio. As already discussed, soluble caseins have a negative impact on the secondary aggregation phase. The result of this study also agrees to this fact because of the decrease in the gel firmness and firming rate as the hydration time increases.

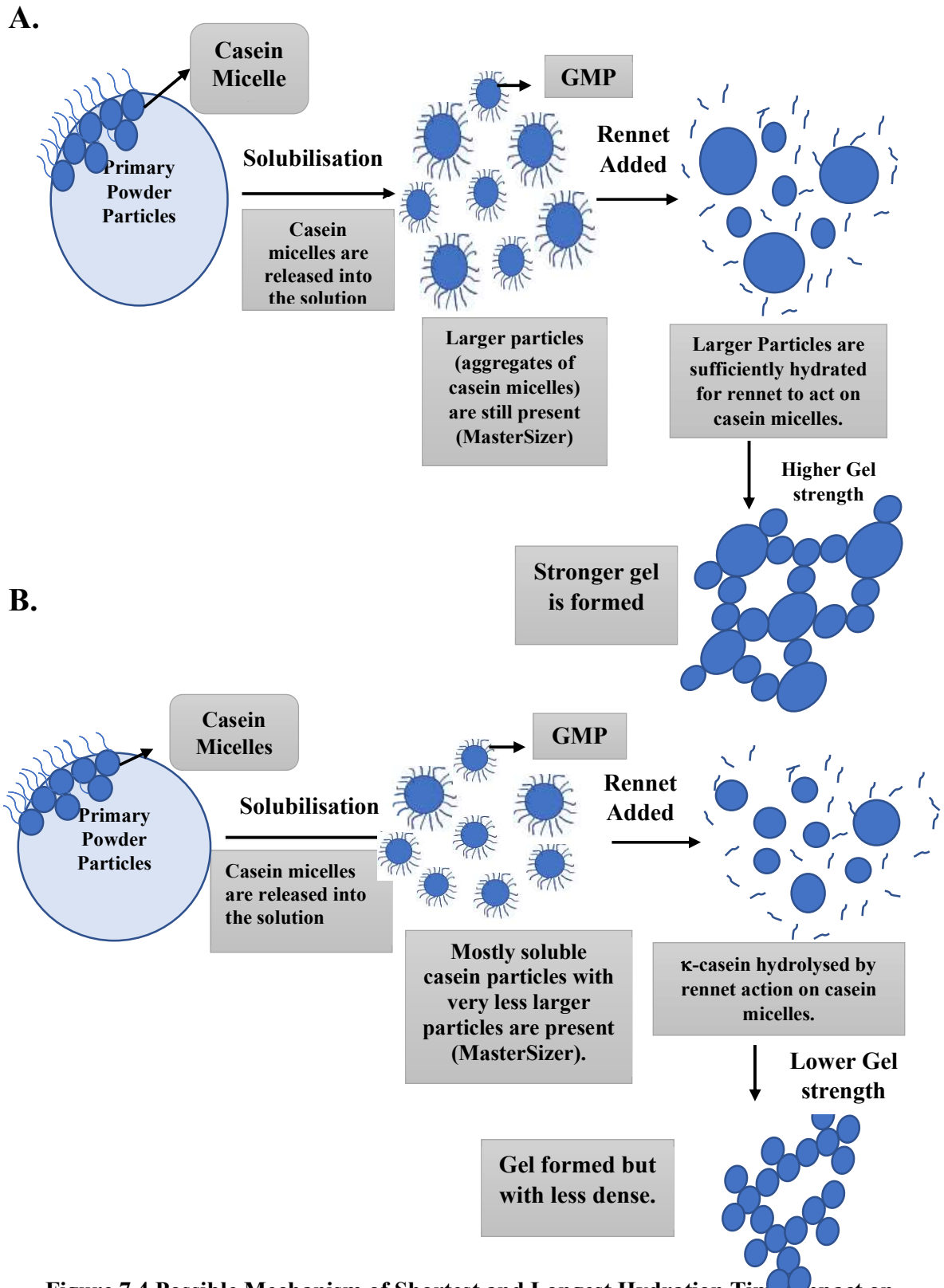


Figure 7.4 Possible Mechanism of Shortest and Longest Hydration Time Impact on Gel Firmness (A) 3 hr Hydration Time (B) 24 hr Hydration Time.

When comparing the longest and shortest hydration time for MCC into the milk base, the shortest hydration time resulted in shorter gelation time with high gel firmness. Figure 7.4 (A) explains the possible mechanism of rennet gelation after 3 hr hydration time. Even though the solubility percentage was 98%, the MasterSizer data indicated large particles are present in the solution. The large particles are likely to be aggregates of casein micelles formed during drying. Higher gel strength indicates the larger particles are sufficiently hydrated for the rennet to have access to the κ-casein protein present on the particle surface. The fully hydrated individual micelles could interact with the larger particles resulting in increased bonds and interactions to form stronger uniform gel structure. If the larger insoluble material was not able to interact with rennetted micelles then they would create gaps in the gel structure and thus weaken it. The insoluble material is likely harder than hydrated micelles thus the higher the concentration of insoluble material the more likely a firmer structure is formed. Figure 7.4 (B) explains the possible rennet gelation mechanism after 24 hr hydration time. The MasterSizer data indicated large particles are still present but comparatively lesser than 3 hr hydration time. Also, the soluble casein is higher in 24 hr hydration time, which slows down the rennet gelation without affecting the function of chymosin. It has been suggested in the literature that casein proteins may interact with rennet-altered casein, providing enhanced steric repulsion (Gaygadzhiev, Massel, Alexander, & Corredig, 2012).

7.6 Summary

In this chapter, the effect of skim milk-fortified MCC solution on the rennet gelation characteristics has been studied by comparing Rheometric and Formagraph analysis and also highlighted the impact of hydration time on sample preparation. The results found that MCC can be the best alternative cheese milk extender by reducing gelation time with higher gel strength in comparison to other casein ingredients. Moreover, the protein fortification of MCC into milk base has a significant impact on the rennet gelation. A recommendation of 3 hr of MCC rehydration gives the optimal gelation properties and it will also be beneficial to the industries by reducing the hydration time during protein fortification. Using both the gel dynamic tests such as Formagraph and LAOR, comparable results were obtained for gelation properties. By analysing the advantage of both methods, Formagraph plays a significant role by processing multiple samples in parallel, which is an essential factor in scientific testing. Above studied results have a particular significance for standardising proteins in cheese making in industrial settings.

8 Effect of Factors Influencing Rennet Gelation Characteristics of Skim Milk-Fortified MCC Solution

8.1 Introduction

From previous studies, it is evident that the rennet-induced milk coagulation process is strongly influenced by the number of factors such as renneting set temperature and pH. These factors are essential as they affect the final cheese yield and rheological characteristics. From the scientific literature, increasing the renneting set temperature from 18ºC till 40-45ºC, the coagulation time decreases and beyond 45ºC, the enzymes become denatured. Also, the coagulum strength improves in the temperature range of 27-32ºC (Fox et al., 2017). Due to the effect of pH on the enzyme activity, decreasing the pH of the fortified solution reduces the rennet coagulation time and also increases the gel firmness. The most significant effect of reducing the milk's pH are the micellar calcium phosphate solubilization, the decrease in the net charge of the casein molecule, and the casein dissociation from micelles (Daviau et al., 2000).

This chapter aims to understand the influence of renneting set temperature and pH on the rennet gelation properties of skim milk-fortified MCC solution. The rennet gelation test using the Formagraph has been carried out with the fresh MCC samples of standard pH (which is 6.7) and adjusted pH 6.3 using 0.1 M HCl with two temperature conditions (30ºC & 32ºC). Based on the results of this experiment, one temperature and pH combination were selected to study the rheological properties of stored MCC samples using Formagraph analysis.

8.2 Results

The samples were prepared as two replicates according to the protocol described in Section 4.2.3 and 4.2.4. The mean and standard deviation of two replicates is described for longer hydration time as 24 hr MCC and for shorter hydration time as 3 hr MCC using Formagraph analysis.

8.3 Influence of Temperature on Rennet Gelation Characteristics of Renneted SMP/MCC Solution

Increasing the renneting set temperature from 30ºC to 32ºC significantly reduced the mean gelation time with a variation of 2 minute for both the 24 and 3 hr SMP-fortified MCC samples. Table 8.1 (I) and (II) explains the influence of temperature condition of 30ºC followed by 32ºC with sample's standard pH of 6.67 on the rennet coagulation properties of fortified MCC samples. As Table (I) and (II) shows the temperature increase resulted in decreased

gelation and optimal cutting time and also increased the gel firmness and gel firmness rate of the fortified MCC samples. Our data is consistent with Fox et al. (2017) who reported for bovine milk samples that an increase in renneting set temperature results in reduced gelation time with an increase in gel strength. Furthermore, as we discussed in the Section 7.5, 3 hr fortified MCC samples showed a shorter gelation time with higher gel strength in comparison to the 24-hr fortified MCC samples owing to temperature variation.

Table 8.1 Influence of Temperature on Rennet Coagulation Characteristics of Fortified Skim Milk/MCC Solution Using Formagraph Analysis.

I.

Temperature 30ºC/Standard pH	Gel Time, RCT (min)	Formagraph Maximum Gel Firmness, a30 (mm)	Formagraph Gel Firmness rate, FGFR (mm/min)	K20, optimal cutting time (min)
24 hr MCC	22.1 ± 0.12	24.7 ± 0.19	0.035 ± 0	28.41 ± 0.32
3 hr MCC	19.1 ± 0.1	38.7 ± 2.08	0.043 ± 0.01	23.76 ± 0.37

II.

Temperature 32ºC/Standard pH	Gel Time, RCT (min)	Formagraph Maximum Gel Firmness, a30 (mm)	Formagraph Gel Firmness rate, FGFR (mm/min)	K20, optimal cutting time (min)
24 hr MCC	19.9 ± 0.2	38.65 ± 1.12	0.041 ± 0.01	24.41 ± 0.32
3 hr MCC	17.44 ± 0.19	49.555 ± 1.09	0.048 ± 0.01	20.97 ± 0.22

8.4 Influence of pH on Rennet gelation characteristics of Renneted SMP/MCC Solution

As shown in Table 8.2, pre-acidifying the SMP-fortified MCC samples from pH 6.6 to 6.3 had a significant impact on rennet gelation properties. Table 8.2 shows the pH variation data with constant renneting set temperature of 30ºC and 32ºC. By looking at the data of Table 8.2, pH reduction significantly reduced the gelation and optimal cutting time twice as fast with higher gel firmness and firming rate. The reduced gelation and cutting time might be due to the reduced electrostatic repulsion between casein micelles at the lower pH values.

Furthermore, unlike the pH 6.7 data, when comparing the data of 24 hr and 3 hr hydration fortified MCC samples at pH 6.3, there is no significant variation among the rennet gelation

properties. In the chapter 7, the suggested possible reason for increased gelation time in 24 hr hydration MCC sample is the higher amount of soluble casein. This solubilised casein protein interacts with rennet-altered micelles providing enhanced steric repulsion (Gaygadzhiev et al., 2012). Interestingly, when the pH is reduced, the change in ionic environment decrease the steric repulsion between renneted micelles. So, it might be a probable reason for no variation in the rennet gelation properties among the 24 hr and 3 hr SMP-fortified MCC samples.

Table 8.2 Influence of pH on Rennet Coagulation Characteristics of Fortified Skim Milk/MCC Solution Using Formagraph Analysis of 30ºC and 32ºC Renneting Set Temperature.

Samples	Gel Time, RCT (min)	Formagraph Maximum Gel Firmness, a30 (mm)	Formagraph Gel Firmness rate, FGFR (mm/min)	K20, optimal cutting time (min)
Temperature 30ºC/pH 6.67				
24 hr MCC	22.1 ± 0.12	24.7 ± 0.19	0.035 ± 0	28.41 ± 0.32
3 hr MCC	19.1 ± 0.1	38.7 ± 2.08	0.043 ± 0.01	23.76 ± 0.37
Temperature 30ºC/pH 6.3				
24 hr MCC	9.10 ± 0.05	66.26 ± 0.18	0.092 ± 0	10.84 ± 0.05
3 hr MCC	9.10 ± 0.42	66.56 ± 1.36	0.094 ± 0.01	10.66 ± 0.49
Temperature 32ºC/pH 6.67				
24 hr MCC	19.9 ± 0.2	38.65 ± 1.12	0.041 ± 0.01	24.41 ± 0.32
3 hr MCC	17.44 ± 0.19	49.555 ± 1.09	0.048 ± 0.01	20.97 ± 0.22
Temperature 32ºC/pH 6.3				
24 hr MCC	7.95 ± 0.16	68.22 ± 0.14	0.107 ± 0.01	9.34 ± 0.23
3 hr MCC	8.37 ± 0.27	68.06 ± 0.7	0.103 ± 0.01	9.69 ± 0.27

8.5 Rennet gelation characteristics of Stored MCC Sample

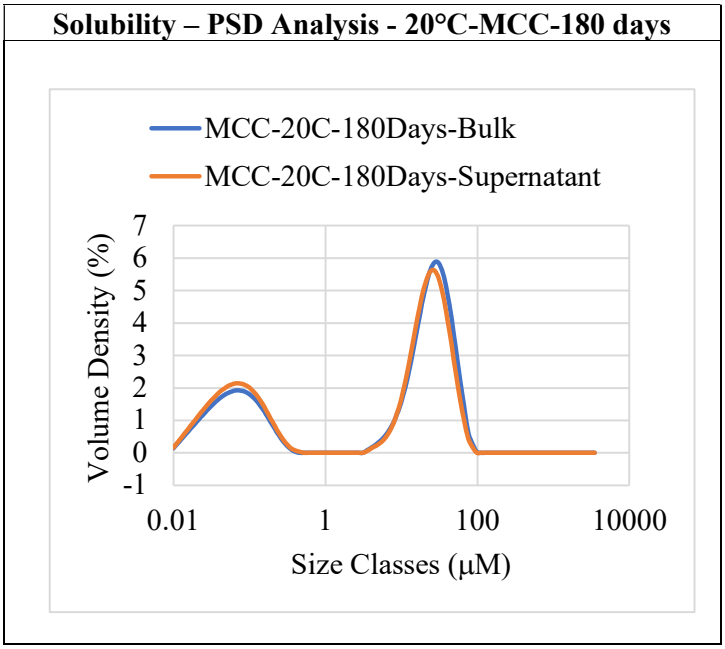
From the data of the previous chapter, the best rennet gelation properties have resulted for 3 hr hydrated MCC-fortified on reconstituted skim milk samples. So, the samples of different temperature-stored MCC were prepared as explained in Section 4.2.4. Using Formagraph analysis, the experimental condition settings were a renneting set temperature of 30°C and pH 6.67. However, we found the best rennet gelation parameters resulted when the renneting set temperature was 32°C and pH 6.3. Due to COVID-19 pandemic lockdown and project's time constraints, five samples were selected to study the rennet gelation property of the stored MCC samples with no modifications done to the experimental set-up conditions such as temperature and pH.

1. From 40°C, one sample stored 36 days was chosen because after which solubility of the samples was completely gone.
2. From 30°C, three samples were selected approximately in the interval of 60 days out of 180 days.
3. From 20°C, one sample was chosen which is stored 180 days.

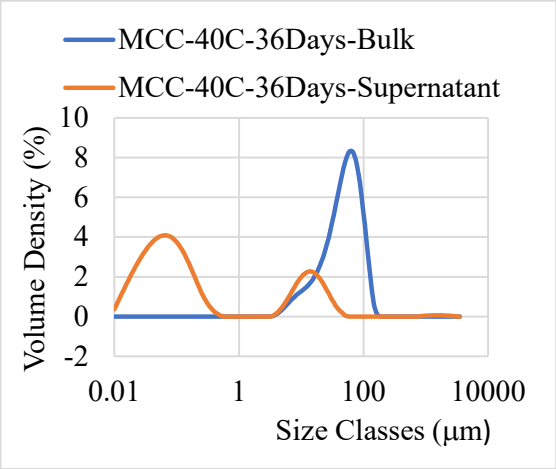
8.5.1 Solubility of Skim Milk-Fortified Stored MCC Samples

The solubility, PSD and pH analysis were examined for the prepared samples and the results are shown in Figure 8.1 and Table 8.3.

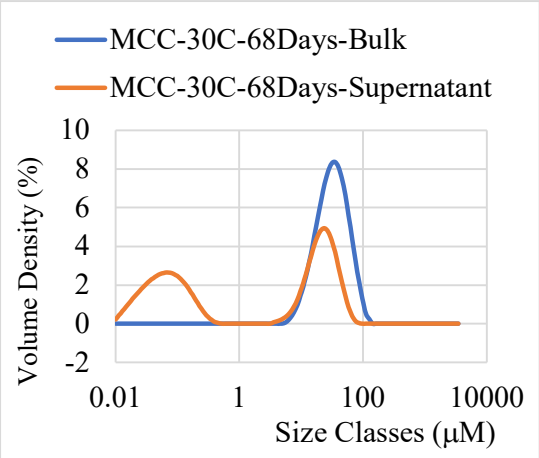
Figure 8.1 PSD Analysis of Skim Milk-Fortified Stored MCC Samples.



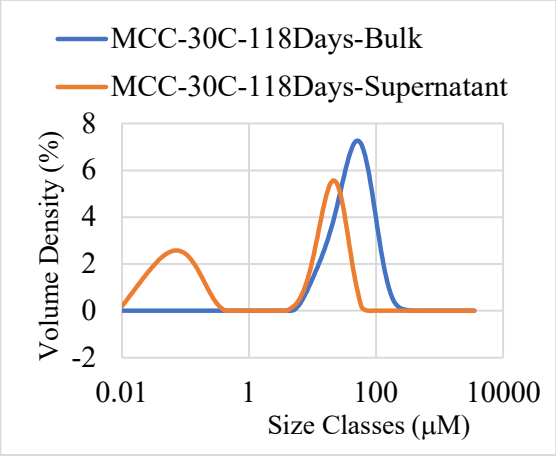
Solubility – PSD Analysis - 40°C-MCC-28 days



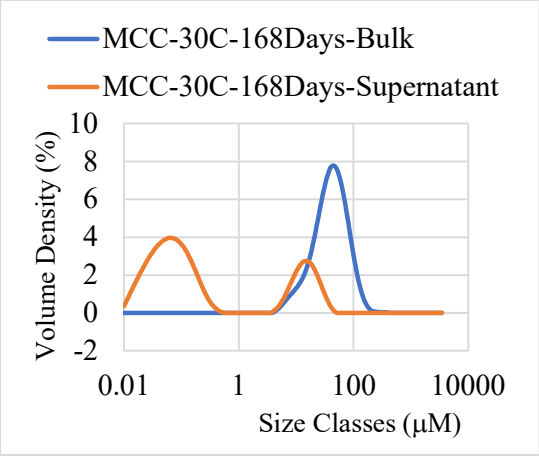
Solubility – PSD Analysis - 30°C-MCC-60 days



Solubility – PSD Analysis - 30°C-MCC-120 days



Solubility – PSD Analysis - 30°C-MCC-180 days



As the study focuses on solubility and its correlation on rennet gelation, the solubility of the reconstituted MCC in a skim milk base was determined. The differences between the solubility of MCC samples reconstituted with skim milk base and water, at pH 6.65-6.70, are highlighted in Table 8.3. From the data, it appears that there is a significant increase in the solubility of stored MCC sample reconstituted with skim milk in comparison to water. However, this could be an artefact as the reconstituted skim milk has 100% solubility in water and further been fortified with a minimal amount of 2.1% of MCC. Therefore, in skim milk, it is likely that the centrifugation-based solubility method is not sensitive enough to pick up changes in

supernatant total solids due to MCC solubility i.e., differences that may be present could be masked by the solubility of the skim milk.

Table 8.3 Percentage Solubility and pH of the Skim Milk-Fortified Stored MCC Samples.

	40°C-MCC-36 days	30°C-MCC-68 days	30°C-MCC-118 days	30°C-MCC-168 days	20°C-MCC-180 days
% Solubility - Skim Milk	78	88	87	81	81
% Solubility – Water	9	62	37	23	82
pH	6.68	6.67	6.66	6.65	6.70

8.5.2 Rennet Gelation Characteristics of Skim Milk-Fortified Stored MCC Samples

Though the solubility of the stored MCC samples apparently significantly increased due to fortification with skim milk, there were still significant differences on the rennet gelation properties. These differences do not seem to be correlated with MCC solubility in skim milk. For example, both 30°C-MCC-168 days and 20°C-MCC-180 days possessed the same solubility in skim and yet their gel firmness values are markedly different 6.24 mm and 19.23 mm respectively. From Table 8.4, we could find there was not a significant difference in the gel time, but there is a visible difference in the gel firmness among the stored samples. It is clear from Figure 8.2, as the storage days increase, gelation time increases coupled with a decrease in the gel strength when comparing the three 30°C MCC samples. In an earlier chapter, we discussed 40°C stored MCC samples lost its solubility after 30 days and 30°C stored MCC samples lost its solubility after 160 days. In this study, we observe a similar trend on the rennet gelation parameters of 40°C-36 days and 30°C-168 days stored MCC. The gelation time occurred at 23-25 minute for all the five samples of the total renneting time 30 mins. The k20 parameter denotes optimal cutting time which was occurring beyond the 30-minute length of the experiment represented as greater than (>) 30 min. The gel firmness rate derived from 1/k20 parameter is similarly represented as greater than 1/30 min (> 0.033 mm/min).

Table 8.4 Influence of Stored MCC Samples on the Rennet Gelation Properties.

Temperature 30°C/pH 6.6/3 hr hydration	Gel Time, RCT (min)	Formagraph Maximum Gel Firmness, a30 (mm)	Formagraph Gel Firmness rate, FGFR (mm/min)	K20, optimal cutting time (min)
40°C-MCC-36 days	25.49 ± 0.36	7.36 ± 1.10	< 0.033	> 30
30°C-MCC-68 days	23.47± 0.81	15.87 ± 2.41	< 0.033	> 30
30°C-MCC-118 days	25.28 ± 0.04	9.54 ± 0.04	< 0.033	> 30
30°C-MCC-168 days	25.81 ± 0.30	6.24 ± 0.54	< 0.033	> 30
20°C-MCC-180 days	23 ± 0.47	19.23 ± 1.27	< 0.033	> 30

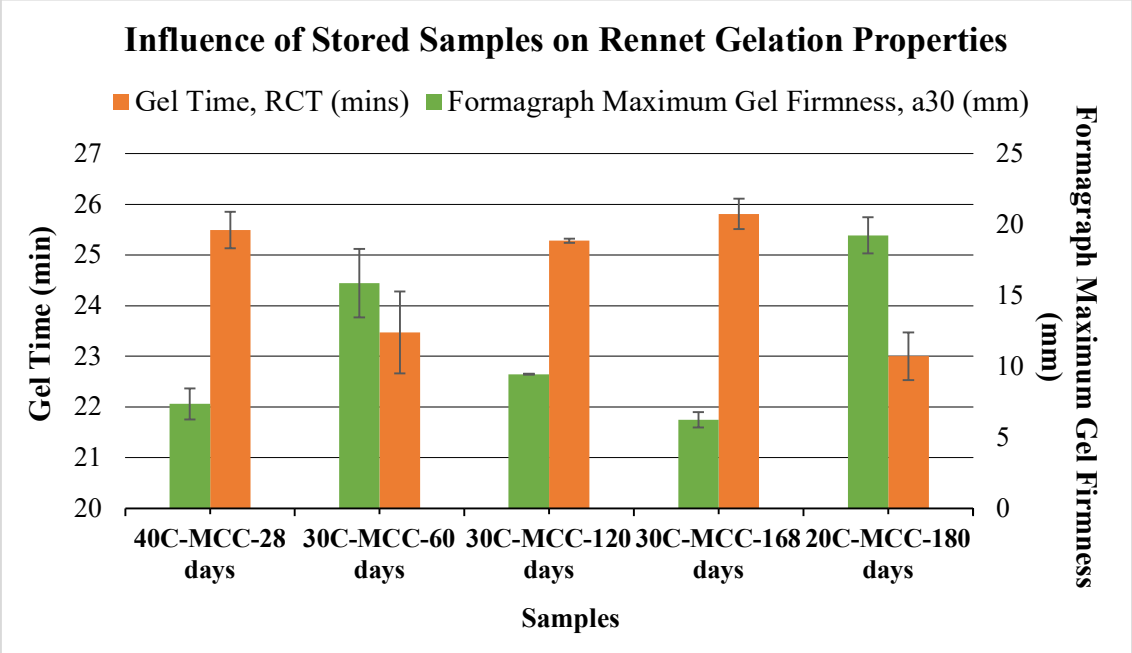


Figure 8.2 Influence of Stored Samples on Gel Time and Gel Firmness.

8.6 Discussion

The effects of renneting set temperature and pH on the rheological properties of rennet-induced skim milk-fortified MCC gels were studied using Formagraph analysis. With an increase in renneting set temperature, the gel strength, a30 and firming rate, FGFR increased, possibly due to higher enzyme activity and faster fusion of casein micelles. It also indicated a higher number of strong bonds with uniform gel network formed between micelles (Mishra, Govindasamy-Lucey, & Lucey, 2005). Sandra et al. (2011) explained the decrease in gelation time with an increase in set temperature perhaps because of an increased casein micelles volume, which

accumulates at a faster rate due to shorter distance between micelles and increased thermal collisions frequency. This action may have contributed to the stronger protein network formation as specified by the greater gel strength at a given renneting time (Orme, 2000). The protein concentration used in this study ~ 5.6% remains constant, which is also said to influence rennet coagulation properties. The present finding also supports Panthi et al. (2019) study which concluded that increasing the renneting set temperature resulted in the rapid formation of firmer gel, which later converts into faster cutting time.

The gelation and cutting time significantly decreased with respect to lower preacidification pH of 6.3 might be due to increased rennet activity (Lucey, 2002) and reduced electrostatic repulsion (Govindasamy-Lucey et al., 2007). Horne (1998) described as the pH decreased, it leads to a decrease in the net negative charge on the casein that reduces electrostatic repulsion force but induces an increase in hydrophobic interactions and electrostatic attraction between micelles. Some solubilisation of colloidal calcium phosphate and therefore an increase in Ca^{2+} activity is also caused by a decrease in pH (Dalgleish & Law, 1989; Lucey et al., 2003). This might be the possible reason for higher gel strength, as it is also indicated by the higher a30 values when pH modified to 6.3.

As mentioned in the literature review, two important reactions occur during rennet gelation; 1) a primary enzymatic cleavage phase 2) a secondary coagulating phase. If one of these two phases are drastically retarded, it might result in weaker gel formation. In the MCC stored samples at 40°C of 36 days, the extended gelation time was delayed by approximately 6 minute, might be due to protein cross-linking, which could limit rennet access to the κ-casein, and so, the rennet took a longer time to diffuse through the skim/MCC solution and therefore a longer time is required until there are adequate renneted casein micelles to create a gel network. The gelation time increases with storage time, and, as such, the primary enzymatic process extended, also impacts the secondary coagulating phase.

Panthi et al. (2019) found that an increased gel firmness and firming rate can be attained by raising the protein content, however, authors reported that fat globules (Lucey et al., 2003) and microparticulated whey protein (Schenkel, Samudrala, & Hinrichs, 2013) acts as an inert (non-interacting) material in cheese and reduce the casein interaction, which further weakens the gel strength. Similarly, the insoluble material of stored MCC could have behaved as unbound or weak interacting material as indicated by the lower a30 gel strength values in the stored samples. And also, the sedimentation of the insoluble material might occur in the stored MCC

sample at the bottom of renneted milk, yielding weaken gel strength (i.e., the protein content during the experiment may not be homogeneous).

Note that this is in contrast to the results for fresh powder in Chapter 7, which showed that decreased insolubility (24 hr hydration data) resulted in decreased gel firmness compared to the same powder with higher levels of insoluble material (3 hr hydration data). The apparent difference in the nature of the insoluble material (i.e., ability to interact with a rennet gel) may lie in the level of hydration of the insoluble material surface layer i.e., the surface layer of insoluble particles from fresh powder is more hydrated than the surface layer of insoluble particle from stored powder.

In the chapter 7, we discussed diafiltration affects the κ-casein hydrolysis and casein micelle aggregation, which can be re-established when reconstituted with skim milk (Zhao et al., 2020). In this chapter, we could also find that the solubility of the stored MCC samples can be improved when recombined with skim milk has shown in Table 8.3. However, there is still variation in solubility percentage according to the storage temperature and time. This small variation between samples resulted in the visible difference between the parameters of rennet gelation. Due to the presence of inert insoluble material in the stored MCC sample, the final gel has a lower gel strength than a sample prepared with fresh MCC sample.

8.7 Summary

In this chapter, the influence of renneting set temperature, pH and different storage temperatures of MCC on the rheological properties of rennet-induced skim milk-fortified MCC was examined. It was found that increasing renneting set temperature from 30°C to 32°C results in faster gelation time and optimal cutting time with high firmness and firming rate. Pre-acidifying to pH 6.3 resulted in faster gelation and cutting time with higher gel strength than the pH 6.7. As the storage temperature and time increases, the MCC solubility decreased, which was correlated with longer gelation times with weaker gel strength. The correlation between solubility and rennet gelation parameters will be discussed in detail in the following chapter.

9. General Discussion

The purpose of this thesis was the study of the effects of storage time and temperature on the solubility and causes for the solubility drop of micellar casein concentrates (MCC) was investigated. Furthermore, the study analysed the effects of storage and hydration on the rheological characteristics of skim milk-fortified MCC solution. This chapter examines the association between the properties analysed from the different experimental methods.

9.1 Correlation between Solubility and Rennet Gelation

The gel time and final gel strength from the Formagraph experiments (Table 8.4) are plotted against the soluble material of the respective MCC sample reconstituted in water and skim milk (Table 8.3) depicted in Figure 9.1 and 9.2. Considering the data presented in both the figures and a correlation coefficient values shows a strong positive correlation between solubility and rennet gelation properties. It indicates that MCC's solubility has a direct impact on the time of the gel formed and, gel structure resulted by enzyme-modified skim milk-fortified MCC samples.

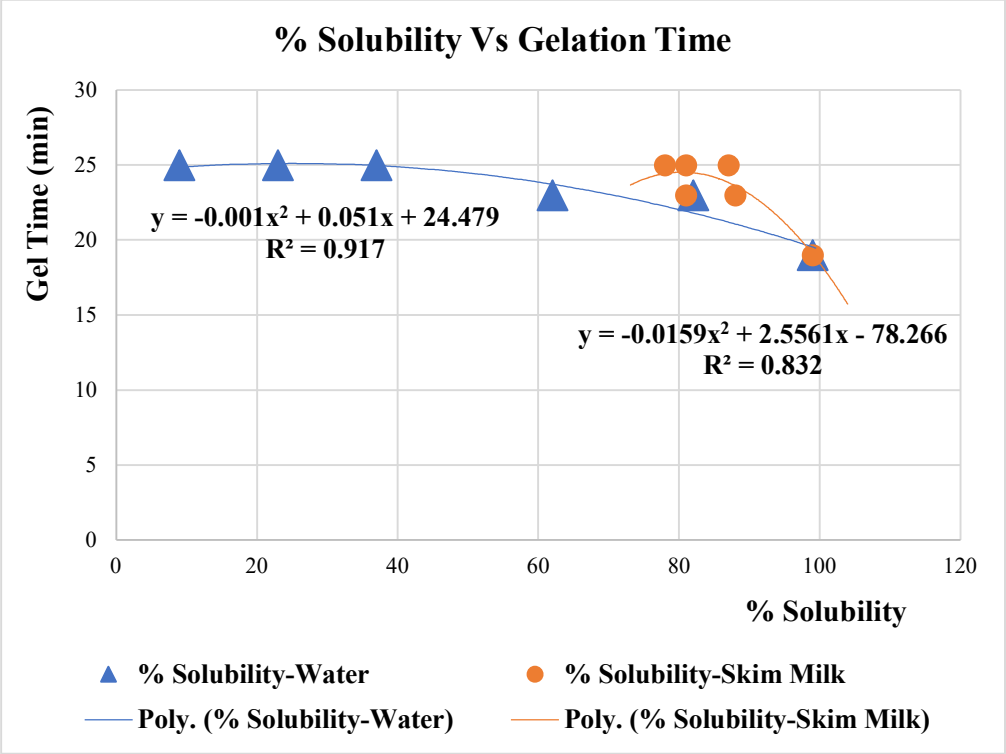


Figure 9.1 Gel Time from Formagraph Experiments as a Function of Solubility of Respective MCC Samples.

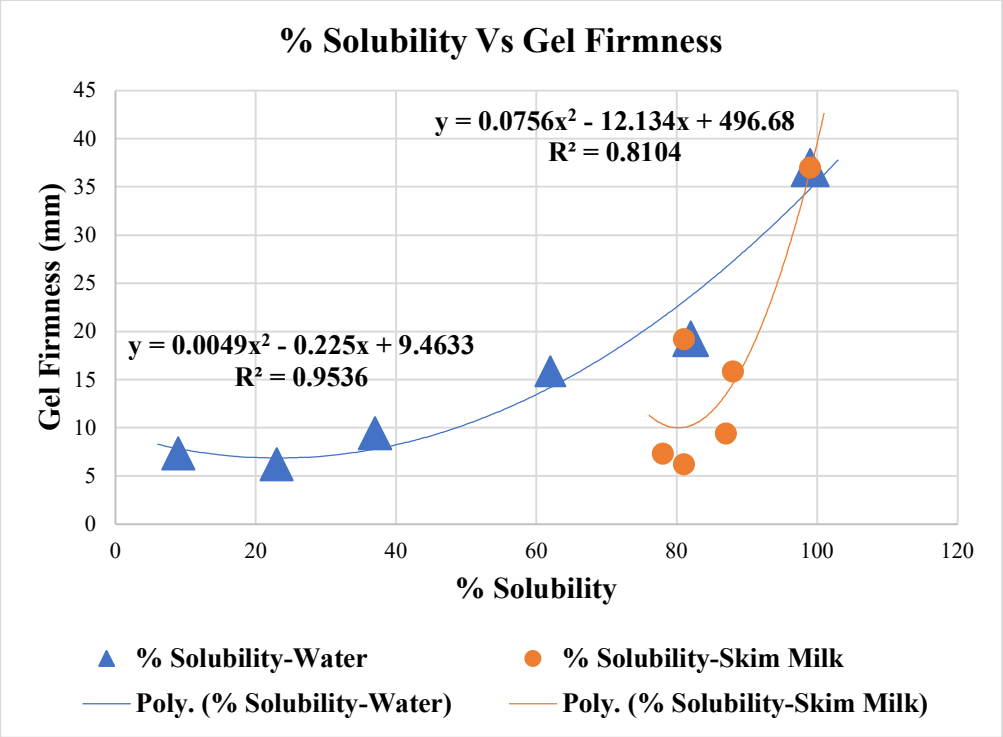


Figure 9.2 Gel Strength from Formagraph Experiments as a Function of Solubility of Respective MCC Samples.

It is important to evaluate the relation between the solubility and rennet gelation properties of MCC samples stored at different temperature so that it can provide insight into the reactions that occur during storage. Also, these equations can be used to predict the rennet gelation properties by determining the solubility of the MCC sample at industrial settings. When comparing the correlation coefficient value of these figures, the MCC solubility reconstituted in water is more than 90%, higher than MCC solubility reconstituted in skim milk. The better correlation between the renneting characteristics of MCC fortified skim milk and the solubility of MCC in water rather than skim milk is likely due to the better resolution of the solubility in water method. The solubility of fresh MCC sample is 99% when reconstituted with water and milk, which resulted in shorter gelation time with higher gel strength. In fresh powder, the solubility is at a maximum, this indicates there is no cross-linking between powder particles, which was also found through colour analysis. In the stored samples, the solubility was slowly decreasing based on the sample's storage temperature and time. The reason might be initially smaller particles completely cross-linked with larger particles partially cross-linked, which eventually leads to complete cross-linking between protein particles resulting in a minimum solubility. For rennet gelation, samples were recombined with skim milk, thereby stored MCC

sample's solubility appeared to increase. But still, there is a variation in the gelation time and gel strength based on the difference in the amount of cross-linking which might occur through Maillard reaction.

9.2 Correlation between Solubility and Browning Index

The correlation between the browning index (Figure 6.1) and solubility in water (Figure 5.3) of the respective MCC powder is presented in Figure 9.3. It appears from the figure's correlation coefficient value, there is a high significance between the soluble material and browning index indicating the increase in browning index value has an effect on the reduction in the soluble nature of MCC powder.

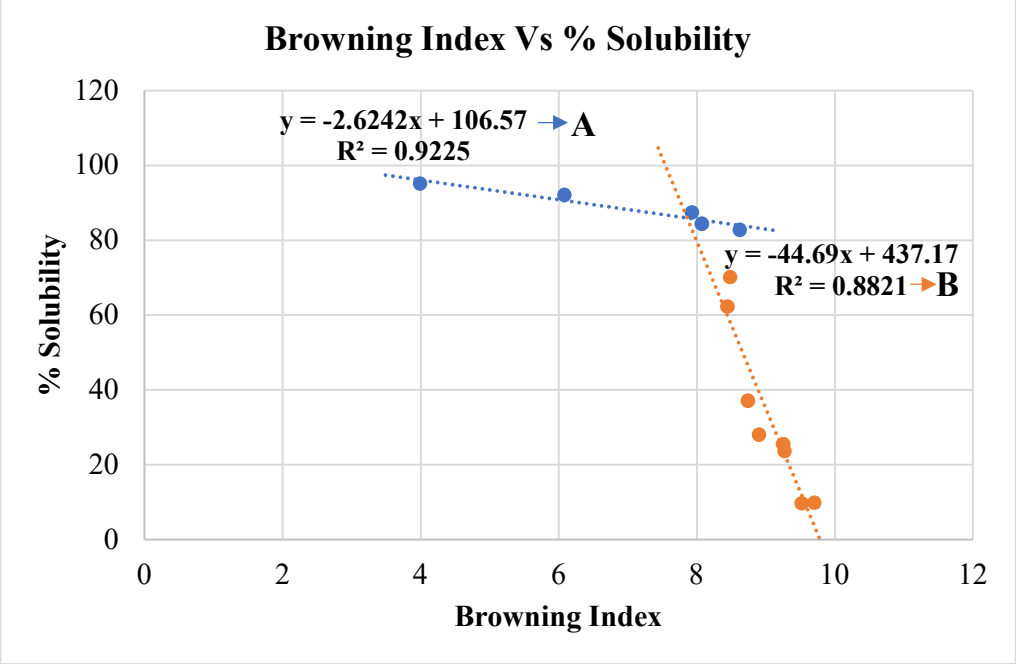


Figure 9.3 % Soluble Material of MCC Powder as a Function of Browning Index Found Using Colour Analysis.

On plotting all the data, it looks like there are two stages of Maillard reaction and protein cross-linking reaction occurs. Initially, in the slow reaction phase, where there is a gradual loss of solubility with cross-linking till the BI value of 8. Once the BI reaches 8, a faster reaction rate occurs when all the protein particles become completely cross-linked, observed by dramatic decrease in solubility. These two variations have been plotted with two linear trendlines in the graph. Both the equation (A & B) of the linear regression model solved to derive the critical browning index, which denotes a change in the solubility as a function of browning can be found as follows,

$$y = -2.6242x + 106.57 \text{ (Equation A)}$$

$$y = -44.69x + 437.17 \text{ (Equation B)}$$

Solving equation (A & B),

$$-2.6242x + 106.57 = -44.69x + 437.17$$

$$-2.6242x + 44.69x = 437.17 - 106.57$$

$$42.0658x = 330.6$$

$$\text{Critical Browning Index, } x = 7.859$$

When a BI is measured as < 7.9, then equation A should be used to predict MCC solubility. If BI is > 7.9, then equation B should be used to predict the MCC solubility.

In the stored MCC samples, the colour analysis is used to form the correlation in Figure 9.3. It could be used as another method for solubility determination and thus, a correlation would be predicted between these effects. Though the centrifugation solubility analysis provides more information about the ratio of powder's soluble/insoluble ratio, it requires twelve hour or overnight to get the results due to the need to dry liquid samples in an oven. In this case, a simple colour test may be used to check protein reaction in the powder, it helps to determine the solubility in couple of minutes. The colour data, via the correlation with solubility could then be used to predict renneting characteristics.

9.3 Summary

In this chapter, the correlation between solubility and various other properties of stored MCC powder at different temperature has been discussed. One of the more significant findings to emerge from this study is that BI through colour analysis can be taken as the key factor to determine solubility, which in turn, is the indicator for the rennet gelation properties of skim milk-fortified MCC solution. The working model generated through this study can be used to predict solubility and rennet gelation characteristics by determining the BI value of the MCC powder through colour analysis. Based on the storage time-temperature profile, data modelling can be done and the final outcome can be used as a template for the industry to predict the product's shelf-life and impact on cheese manufacture.

10.Conclusions and Future Work

10.1 Conclusions

The following conclusions can be summarized from the present study:

1. The solubility of the stored MCC powder in comparison to the fresh MCC powder decreased with the decrease being dependent on the storage time and temperature.
    a) The maximum solubility for the reconstitution of fresh MCC powder can be attained at the rehydration temperature of 50°C with the hydration time of 30 min.
    b) In the overall period of 6 months, the rate of solubility declined: -
        - Almost completely in a month for 40°C stored MCC powder.
        - In 6 months for 30°C stored MCC powder.
        - While only a slight decline rate was noted for 20°C stored MCC powder.
    c) The correlation between solubility and various other properties has been studied and found solubility can be used as an indicator for rennet gelation properties.
2. The colour analysis revealed the reason for the decrease in the solubility of stored MCC samples was likely due to the protein cross-linking and Maillard reactions.
    a) Though there is a low lactose content in the MCC powder, the increase in the browning index indicates the Maillard reaction does still occur in the powder particles was dependent on the storage conditions.
    b) Browning index can be used as a measure to predict the solubility – a working model was produced in the Section 9.2.
    c) The microstructural analysis of SEM and TEM gave a good insight of particle size, shape and a cross-sectional view of protein-protein interaction of the MCC powder.
3. The single-stage high-pressure homogeniser was found as an effective solution to fully solubilize MCC powder and overcome the negative effect of storage conditions.
    a) A single-stage shear force pressure of as low as 100 bar is sufficient to break the powder particles in comparison to single-stage pressure of 150 and 200 bar.
4. The impact of hydration time on the sample preparation of the skim milk-fortified MCC solution provided significant information.
    a) In comparison to 24 hr hydration time, the study recommends 3 hr hydration as it results in faster gelation and cutting time with higher gel firmness and firming rate which will also be beneficial to industries by reducing the sample preparation time during protein fortification.

b) Two gel dynamic tests such as Formagraph and LAOR have been compared and found both the methods can provide comparable results for gelation properties, but, the Formagraph had an advantage of assessing 10 samples at a time.

5. The influence of renneting set temperature and pH on the rennet gelation properties of the skim milk-fortified MCC solution gave a valuable insight into the experimental conditions for the production of cheese.
    a) Increasing in renneting set temperature from 30°C to 32°C resulted in faster gelation and cutting time with an increase in the gel firmness and firming rate.
    b) Decreasing the pH of the solution from 6.7 to 6.3 reduced the gelation and cutting time twice as fast with an increase in the gel firmness and firming rate.
6. Formagraph gelation analysis shows that the physical properties of renneted skim milk-MCC gels have been influenced by the properties of MCC powder stored at different temperatures of 20°C, 30°C and 40°C.
    a) As the storage temperature and time increases, the solubility decreased, which reciprocated in the longer gelation and cutting time with weaker gel formed.
7. Measuring the browning index of MCC powder looks like a promising method to predict both solubility and rennet gel characteristics.

10.2 Recommendations and Future Study

It is recommended that further research can be undertaken in the following areas:

1) The experimental investigations are needed to estimate the cause of drop in solubility with reconstitution temperature above 60°C.
2) Based on the storage time-temperature profile, data modelling can be done and outcome can provide a useful template for logistics team to predict the product's shelf-life in a commercial setting.
3) Further studies should be carried out to find the impact of dry versus humid storage conditions of the sample powders. As in this study, the samples were enclosed in the airtight bags during storage experiments.
4) Further method development for the microstructural analysis of SEM and TEM is required to visualise the clear picture of extra skin layer formation, if it occurs, around casein micelle of MCC powder.
5) Validate the results of gelation time and gel firmness with homogenization step to reduce the negative effect of storage time and temperature.
6) If homogenizer produces the same result as a good fresh control MCC on a temperature and time abused MCC powder, then powder storage is not critical for product consistency, else recommend temperature-controlled storage.

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12. Appendix

A. Flowchart of 24-hr and 3-hr Sample Preparation of Skim Milk-Fortified MCC Sample for Rennet Gelation Testing