IMPACT OF DIRECT STEAM INJECTION PROCESSING ON THE FUNCTIONALITY OF MILK PROTEIN CONCENTRATE

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Abstract

The global demand for the milk protein concentrate (MPC) formulated consumer products and MPC as an ingredient has grown over the last decade. The U.S. still imports significant amounts of MPC, even after doubling its domestic MPC production over the past few years. Milk protein concentrates and isolates provides a variety of food ingredients, which gives excellent functionality and nutrition to different foods and beverages. However, caseinates and whey protein concentrates have good functionality, especially solubility, as compared to the MPCs. The solubility of MPCs decreases with time. The objective of this thesis is to study the combined effect of the direct steam injection process and pH change on the functionality of MPCs.

The functional and compositional characterization of MPC 70, 80 and NFDM, manufactured using combined effect of 7, 8, and 9 pH levels at 85 and 105°C direct steam injection (DSI) temperatures, has been reported in this thesis. High solubility samples of MPC 70, 80 and NFDM, were analyzed for solubility and microstructure after storage period of 20 days at 40°C. Proximate composition, protein compositional analyses, zeta-potential, hydrodynamic diameter (d_h or d_{err}), functionality (solubility, foaming, viscosity, heat stability, and rennet gelation), and microstructure analyses were performed to understand the effect of treatment on powders. Our hypothesis was that pH and temperature changed configuration of casein and whey proteins, respectively, and resulted in different functionality of MPCs. We have observed an increase in the means of solubility, zeta potential, ash, viscosity, and a decrease in hydrodynamic diameter of MPC70 and 80 samples with increased pH at both evaluated temperatures. By using scanning electron microscopy, a decrease in aggregation was observed with an increase in pH at both temperatures in M70,

and M80 samples. The opposite effect was seen in NFDM samples. No significant effect was observed in protein compositional, foaming, fat, protein, and moisture analysis in NFDM, MPC70, and 80 samples.

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Dedication

This work is dedicated to my parents, who have been my force to work towards my dreams,

and

my friends in Moscow, Idaho, US who have always supported me and made me feel at home during my master's degree.

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Chapter 1: Introduction

In the past few years, the growth in demand for high protein ingredients has led to an increase in the global demand and, thus, the production for milk proteins and milk protein concentrate (MPC) powders. MPCs contain both casein and whey protein in a ratio similar to that of skim milk. They are high in protein and low in sugar and minerals (Sikand et al., 2011; Uluko et al., 2016). Functional properties including, emulsification, gelation, thickening, heat stability, foaming, and water absorption, has led to the increased use of MPCs in different food products (Singh, 2007; Ye, 2011; Meena et al., 2017). Solubility of MPCs decreases during storage time, which hinders their application in many products (Meena et al., 2017). Improving the solubility of MPCs will broaden their use in different food products. Direct steam injection (DSI) at 107°C and pH 9 enhanced the functionality of the combination of rice and pea proteins (Pietrysiak et al., 2018). The goal of this study was to check the impact of DSI on MPC functionality, with an objective of determining the combined effect of three pH levels (7,8, and 9), at two DSI temperatures (85 and 105°C) on the functionality and physiochemical properties of MPC70, MPC80, and non-fat dry milk (NFDM). This project will help the dairy processors to manufacture the MPCs for specific functionalities and use those MPCs in desired food products. Chapter two of this thesis summarizes the production, functional properties, and approaches to increase the solubility of MPCs. Following that, chapter three describes the study of DSI treatment on MPCs.

Chapter 2: Literature review

2.1 Introduction

Milk contains high-quality milk proteins: caseins and whey proteins in the ratio of 80:20 (Patel et al. 2015). Both of these proteins provide all the essential amino acids which fulfill the protein requirement for various functions in the body (Bos et al. 2000). Due to these reasons, milk proteins are considered as a standard reference protein to determine the nutritive value of food proteins (Miller et al. 1999).

Total global milk production is estimated to grow up to 981 million tonnes by 2028 (IDF, 2019). India is the biggest global milk producer (22% of global production), followed by the United States of America, China, Pakistan, and Brazil. Countries like New Zealand, the United States of America, Germany, France, Australia, and Ireland have a surplus of milk. However, China, Italy, the Russian Federation, Mexico, Algeria, and Indonesia are milk deficient countries (FAO, 2020). In 2021, milk production in the United States was approximately 225,000 million pounds, an increase of 13 % over the past ten years (USDA, 2022).

In 2020, California was the leading producer of milk (18.49% of the United States production), followed by Wisconsin and Idaho (USDA, 2022). The demand for milk proteins is increasing globally due to the growing demand for high protein ingredients. Concentrated milk proteins act as emulsifiers, flavor enhancers, flavoring agents, formulation aids, humectants, stabilizers and thickeners, texturizers, and sources of high-quality protein (USDEC, 2018).

Milk protein concentrates (MPCs), and isolates (MPIs) powders have high-quality

proteins with their high nutritional and functional properties (Agarwal et al. 2015). They contain both casein and whey proteins in the same ratio as present in the milk (Agarwal et al. 2015). MPCs and MPIs are manufactured by ultrafiltration (UF) of pasteurized skim milk and followed by spray drying of retentate, which is obtained from UF process. Whey proteins are mostly present in their native states because the heat load is kept minimum throughout the MPC production (Agarwal et al. 2015). However, milk powders obtained through high heat treatment like evaporation have more whey protein denaturation as compared to the MPCs because whey proteins are heat sensitive, and casein is a pH-sensitive protein in milk (Meena et al. 2017). In this chapter, literature related to the MPC production and issues related to improving its functionality is reviewed to get a better understanding of the MPC research, and the gaps needed to fill the MPC research.

2.2. Approximate composition of non-fat dry milk, milk protein concentrate, and isolates

The protein content of milk protein concentrate (MPCs) is generally depicted in their name, for example MPC70 means it has 70% protein content on the dry matter basis. The protein content of non-fat dried milk (NFDM) powder varies from the 34 to 36 % of total solids, whereas the protein content of MPC and MPI powders varies from 42 to 85 %, and ≥ 90% of total solids, respectively, (Patel et al. 2014). Nevertheless, most commonly used MPCs are MPC 42, MPC 70, MPC 80, MPC 85, and MPI (Patel et al. 2014). MPCs are further classified into three types on the basis of their protein content: low (≤40%), medium (60-70%), and high (≥80%) protein powders (Sikand et al. 2011).

The MPCs and MPIs have the casein to whey protein ratio equal to the 80:20, and

which is equivalent to the casein to whey protein ratio of the original milk. With the increase in the protein content in the MPCs, the lactose content decreases because more lactose is removed in the permeate with the help of ultrafiltration and diafiltration (optional: used in the manufacturing of MPCs with high protein content) to get the high protein content in the MPC. However, ash content almost remains unchanged because some content of it is removed in the permeate, and the bound mineral content is concentrated in the retentate with protein (NFM, 2021) (Meena et al., 2017).

2.3. Production of milk protein concentrate

For MPC production, skim milk is concentrated with the help of ultrafiltration (UF), and UF retentate is obtained with the desired protein and TS ratio and followed by the evaporation (optional) and spray drying of the retentate to get the MPC in powder form. Generally, a very mild heat treatment, like high-temperature short time (HTST) pasteurization, is given to the skim milk at its native pH, so that MPC should have undenatured casein and partially denatured whey proteins. Many functional properties depend upon protein stability and solubility, and these properties are poor in the case of MPCs, because of their high calcium and protein content (Meena et al., 2017). Therefore, different technical approaches are being used to improve the solubility and emulsification capacity (protein stability) of MPCs.

2.3.1. Raw material and its heat treatment

The raw material of MPC production is skim milk with a good microbial quality (low total and spore counts) (Cassano et al. 2014). Therefore, some researchers suggested using microfiltration as a pretreatment of skim milk. Skim milk is obtained by cream separation from the whole milk, and therefore, compositional aspects and physio-chemical properties of

whole milk are usually considered. The approximate composition of milk, physio-chemical constants, and protein fractions in milk are given in table 2.3.1.1, 2.3.1.2, 2.3.1.3. respectively (Fox and Mcsweeney 2015) (Walstra et al. 2006). Skim milk is usually heated to 72°C/15 s (HTST pasteurized) for inactivating the undesirable microorganisms and enzymes in it. As casein is not much affected by the pasteurization temperature, but the whey protein does, therefore, heat load provided to skim milk is kept minimum to keep the denaturation loss of whey proteins as minimum as possible. Moreover, federal agencies like European Food Safety Authority (EFSA) (EFSA, 2016), and Food Safety and Standards Authority of India (Koutchma, 2018) have approved the UV treatment of milk, at the place of pasteurization, to improve the nutritional valve of milk. Therefore, the non-thermal technologies, like UV-C system, could be used at the place of pasteurization to avoid the heat denaturation of whey proteins in the production of MPCs (Pendyala et al., 2022; Vashisht et al., 2022).

Table 2.3.1.1 The approximate composition of milk

Component	Average content in milk (% w/w)
Water	87.1
Solids-not-fat	8.9
Lactose	4.6
Fat	4.0
Protein*	3.3
Casein	2.6
Mineral substances	0.7
Organic acids	0.17
Miscellaneous	0.15

* Non-protein nitrogen compounds not included.

Table 2.3.1.2 Physio-chemical constants for milk

Physio-chemical constants	Value
Specific gravity (20°C)	1.030
Viscosity (20 °C)	2.127 mPa s
Surface tension (20°C)	52 N/m
Titratable acidity	0.14 to 0.16%
pH	6.6
Redox potential (25°C, pH 6.6, in equilibrium with air)	+0.25 to 0.35 V
Thermal conductivity (W/m K)	0.559

Table 2.3.1.3 Protein fractions in milk

Protein	Quantity in g/kg of milk
αs1-casein	10.7
αs2-casein	2.8
β-casein	8.6
κ-casein	3.1
β-lactoglobulin	3.2
α-lactalbumin	1.2
Serum albumin	0.4
Lactoferrin	0.1
Immunoglobulin	0.8

2.3.2. Ultra-filtration (UF), its membrane and modules

There are four pressure driven membrane filtration processes: UF, nano-filtration, micro-filtration, and reverse osmosis. UF separates the feed molecules based on their charge, sizes, shapes, and affinity towards the membrane (Aimar et al. 1988). UF selectively sieves the feed components based on their molecular weight. Membranes are usually described by their pore size and molecular weight cut-off (MWCO). MWCO means the lowest molecular weight of a molecule that is 90% retained by the membrane. Generally, UF membranes which are having the sharp MWCO are used as compared to the membranes having diffused MWCO. 1-50 nm is a pore size range for the UF, and the operating pressure for UF varies from 0.01- 0.1 kPa. Moreover, UF works optimally at the transmembrane pressure (TMP) of 1 bar, where TMP is a pressure gradient that exists through the membrane, from the feed side to the permeate side at each point along the membrane surface. UF retains the colloidal milk components like caseins, whey proteins, micellar salts and residual fats and permeate of the

UF process includes water soluble components like lactose, salts, non-nitrogen, and vitamins. The molecular weight and diameter of milk constituents are shown in table 2.3.2.1. (Walstra et al. 2006) (Kessler, H.G. 1981).

Table 2.3.2.1 Molecular weight and diameter of milk constituents

Milk constituents	**Molar Mass**	**Diameter (nm)**
Water	18	0.3
Calcium	35	0.4
Lactose	342	0.8
αs1-casein	~23600	10-600
αs2-casein	~25200	
β-casein	23983	
κ-casein	~19550	
γ-casein	~20500	
β-lactoglobulin	18283	4.0
α-lactalbumin	14176	3.0
Serum albumin	66267	
Proteose peptone	4000-40000	
Immunoglobulin (Ig)- IgG1, IgG2	~150000	
IgA	~385000	
IgM	~900000	
Lactoferrin	86000	
Transferrin	76000	

Tubular ceramic UF membranes with spiral-wound configuration are generally used

for the MPC production, with the processing variables like TMP of 200-400kPa and permeate flux of 30 to 120 L/h/m². In high viscous concentrations, tubular membranes and plate and frame modules can be used. (Cassano et al. 2014). The widely used membrane materials for UF are polysulphone (PS), polyethersulphone (PES), and ceramic membrane materials. Third generation membranes like mineral membranes/ ceramic membranes are generally used in UF, and these are made up of alumina, zirconium, silver or carbon on microporous support of the same material. Advantages of these membranes are they can withstand high temperature (up to 400°C), operating pH range is 0 to 14, operating pressure can be up to 2.0 MPa, and these membranes have a long membrane life. Moreover, these membranes are easy to clean and onsite replacement is also easy. These membranes are high cost, availability only in the tubular configuration, and large pore size which can restrict the use of ceramic membranes. Generally, in industry, the UF process is operated either at ~ 10°C or at ~ 50°C, but both temperature points have advantages and limitations with those.

2.3.3. Diafiltration (DF) and permeate removal

With an increase in the protein content in the UF retentate, the viscosity of retentate increases, and chances of concentration polarization and fouling increases. So, in diafiltration, a known quantity of water is added in the UF retentate and properly mixed which leads to more concentrating of retentate by more removal of water-soluble components like lactose and salts, and thus the viscosity of retentate increases. UF can increase protein content, but not enough for certain products and therefore DF is combined with UF to get higher protein concentration in UF retentate. DF is generally used to get the protein content of final powder higher than 65g/100g, with a decrease in the lactose content (<5g/100g) in the retentate (Cassano et al. 2014).

2.3.4. Evaporation (optional) and spray drying of skim milk retentate

Prior to spray drying, evaporation may be done with one- or two-stage falling-film tubular evaporator to further increase the UF retentate solids, and so, to get the required physical characteristics, like bulk density, of spray dried powder (Cassano et al. 2014). But some adverse changes in the product quality like off-flavor development, protein denaturation due to high heat treatment and are caused by the applied evaporation. Moreover, evaporation decreases the solubility of MPCs because of the denaturation of whey proteins.

In the spray drying process, UF retentate is sprayed into small droplets in a heated chamber with the help of pressure or rotary atomizer, and inlet air and outlet temperatures are kept 180°C and 85°C, respectively. There is a formation of small sized powder particles due to instant removal of moisture at a higher temperature in the chamber.

2.3.5. Packaging and storage of MPC

Packaging is done to prevent food from external contamination and to keep it safe for human consumption. Good packaging of the product also contributes to enhancing the shelf life of the food product. The major considerations required during the selection of the packaging material for dried milk products are the absorption of moisture, oxygen uptake, uptake of odor and bacteria and exposure to light (Cummius, 1976). The packaging material requirements for the MPCs are similar to skim milk powder packaging material because of the presence of very less fat in both powders. But the considerations for water vapor barrier are made, otherwise, the physical and functional properties of MPCs are negatively affected by water vapor absorption. Therefore, packaging material used for the whole milk powder and skim milk powder packaging can be used for the packaging of MPCs, like kraft-

paper/polyethylene, kraft-paper/nylon-polyethylene laminate, aluminum polyethylene laminate, and plastic bags etc.

2.4. Production of NFDM

Skim milk powder (SMP) and nonfat dry milk (NFDM) both are very similar products and produced with the help of evaporation and spray drying which is done for the removal of water from the pasteurized skim milk. Both products should have moisture and milkfat less than or equal to the 5% and 1.5% by weight, respectively, however, the only difference between these products is that SMP should have minimum 34% milk protein content, but no such standards are available for the NFDM. According to the whey protein nitrogen index, NFDM are classified into 3 types: low-heat, medium heat, and high heat powders (USDEC, 2022).

2.4.1. Evaporation

Total solids of pasteurized skim milk are concentrated up to 40 to 48% using the evaporator. Refractometer is used for continuous checking of total solids. To produce the low-heat NFDM, double and triple effect evaporators should be used carefully to prevent whey protein denaturation. On getting the desired concentration of total solids, for further processing, condensed skim milk is heated to 61.6 to 68.3 °C because it will decrease the viscosity of condensed skim milk and thus it will flow easily in the atomizer for spray drying (ICAR, 2022).

2.4.2. Spray drying

In spray drying, the high-pressure pump is used to push the hot product into the spray nozzles and then mist like droplets are formed in the drying chamber. The pressure of the pump depends upon the nozzle design and size, inlet and outlet temperature, drying chamber

characteristics, particle size, and moisture content desired (ICAR, 2022). Direct flame or steam coils can be used to heat the input air to a temperature of 120°C to 260°C. Moisture of the NFDM is controlled by the exhaust air temperature, but changes can also be done through adjustment of inlet air temperature. When air is humid then a supplementary heater or re-drier can be used after the primary drying chamber to get the required moisture in the last product (ICAR, 2022).

2.4.3. Packaging and storage

The packaging material and storage conditions of NFDM is same as for the MPC powders. The packaging of hot product: i) may result in lump formation due to heat caking and ii) can also cause the formation off flavor and off flavor increases rapidly during storage. To prevent these defects, a 25-mesh screen with No. 36 wire gage is usually used before the packaging of NFDM. (ICAR, 2022).

2.5. Effect of processing parameters on calcium removal and membrane flux

Ultrafiltration of skim milk is usually done at either temperature ≤ 10 °C or in between 40-50 °C. (Liu et al. 2014). At lower temperature (9 to 12°C), bacterial growth is minimal in the UF retentate for but the permeate flux is low, while at higher temperature, both bacterial growth and permeate flux are having high values as compared to the low temperature operating conditions (Cassano et al. 2014).

According to the Cheryan (2018), viscosity of retentate decreases with the increase in the feed temperature and thus permeate flux through the UF increases, but at a lower temperature of ultrafiltration, more calcium passes through the membrane because it is more soluble at lower temperature, whereas the flux rate will be very low at lower temperature. The viscosity of retentate also increases with the increase in the protein concentration which

decreases the membrane flux of UF system. (St-Gelais et al. 2010). Membrane flux decreased with an increase in operating pressure (Babu et al. 2001). Membrane flux increases with an increase in diafiltration water (Gavazzi-April et al., 2018).

The maximum dissolution of casein occurs at pH 5.1 and 5.4 with temperature 4°C and 20°C, respectively (Dalgleish and Law 1988). Membrane flux drop downs due to a decrease in pH, however, minerals are removed easily in permeate, with the dissolution of casein micelle due to the decrease in pH (St-Gelais et al. 2010). When UF is done at the 3 temperatures: 15, 30 and 50 ° C, then researchers noticed that Calcium removal is less at 50 ° C, and more at 15° C, and which resulted in the decreased fouling of membrane at lower temperature (15° C) as compared to the higher temperature (30 and 50° C) (Luo et al. 2015).

2.6. Functional properties of MPC

According to the Figueira (2018), functional properties confirm the required qualities of the product, commonly by interacting with other food components using the proper concentration of the selected components and under the appropriate conditions. Nutritional and functional characteristics are responsible for the high importance of the MPCs, in the food formulations (Patel et al. 2014). The components and interactions which affect the functional properties are hydrogen bonding, hydrophobic interactions, ionic forces, and covalent bonding between solvent, ions, proteins, saccharides, lipids, and other components of the provided product (Damodaran et al. 1997). Poor solubility is the major concern of the commercial MPCs, and solubility decreases with storage period (Meena et al. 2017; Sharma, 2021). Functional properties of MPC include water binding, thickening and viscosity, emulsification, foaming and whipping, gelation, heat stability, and color development.

2.6.1. Heat stability

Milk and milk powders should be heat stable because milk and milk products undergo heat treatment to enhance the shelf life and ensure their safety for human consumption (Fox 2010). Heat stability is a non-coagulating or gelation ability of milk at high processing temperatures, and the time that elapses between the placing of milk sample in oil bath at 140 C (for liquid milk sample) or 120 C (for concentrated milks/membrane retentate) to the onset of visible coagulation is known as heat coagulation time (HCT) (Meena et al. 2017). It is usually measured by the heating the powder dispersion at 140 C in oil bath until heat coagulation is visually observed.

2.6.2. Solubility

Hydrophobic and hydrophilic properties are responsible for the solubility of the proteins. pH is the one of the key factors responsible for the protein solubility in aqueous. Net charge on protein molecule is 0 at the isoelectric point and as we move away from the isoelectric point means towards the higher pH then ionic hydration and electrostatic repulsion is increases and hydrophobic interaction in-between the non-polar region decreases (Damodaran et al. 1997). Processing parameters like evaporation and any other heat treatment may cause the denaturation, and subsequent cross linking and aggregation, and thus decreases the solubility of MPC (Figueira 2018). MPC solubility is a key driver factor for other functional properties (Meena et al. 2017). Surface properties of the casein micelles are more responsible, as compared to the interiors of the micelles, for the stability of micelles. -13 mV zeta potential (20 °C) on casein micelles is present due to the dissociated carboxyl, and which are hydrophilic in nature and some ester phosphate groups, and this negative charge is responsible for the electrostatic stabilization of the casein micelles. Removal of this

layer with some treatment such as pH adjustment causes the aggregation of casein micelles. Hydrophobic and electrostatic bonds are responsible for the association of the casein micelle from inside. Colloidal calcium phosphate (CCP) bind to serine phosphate groups of casein therefore plays an important role to electrostatically bind groups of caseins together(Fox et al. 2015). Zeta potential and particle size is calculated and correlated to check the stability of casein micelle (Meena et al. 2017).

2.7. Problem of poor functionality of MPC, and its mechanism

2.7.1 Solubility and mechanisms of insolubility development in MPC

Solubility is an important functional property of the protein rich powders and other functional properties like gelling, foaming, emulsification, thickening, water absorption, and heat stability etc., are interconnected with it. Ultrafiltration, diafiltration, and spray drying changes the salt equilibrium of the colloidal and soluble phases of the calcium and phosphates in the protein stabilization system, and which may lastly have a negative impact on the milk protein environment (Meena et al. 2017). Solubility is adversely affected by this kind of alteration. MPCs with a very high protein content are reported to have poor solubility especially under the storage conditions of high moisture conditions and with temperature more than ambient one, which restricts their use in a lot of potential food applications (Patel et al. 2014; Sharma, 2021).

The solubility of MPCs is poor at 20°C, but it increases with increase of temperature. According to the (Meena et al. 2017), many mechanisms are given to explain the insolubility of MPCs and some of them important ones are:

i. Proteins forms a kind of barrier ($\alpha_s - \beta - Cn$) along with the surface of MPC powders, which hinders the water transportation and thus hinders the hydration of MPCs (Mimouni et al., 2010).

ii. Residual fat covers the surface of the MPC particles (Fang et al., 2012; Khalesi et al. 2021).

iii. Cross linking of casein micelles prevents the MPCs from dispersing (Anema et al., 2006).

iv. Mimuni 2009 reported that despite the large quantity of insoluble particles, the slow dissolution kinetics is responsible for poor solubility, and it depends upon both temperature and agitation.

(Mckenna, 2000) stated that after 6 month of storage time, MPC 85 has insoluble portion includes the large size particles of approximately 100 mm, especially of casein micelles, which are bonded through some protein-protein interactions, and these particles are not soluble even when dispersion is made at 45°C for 30 minutes. The bonds which are responsible for these kinds of interactions are both covalent (which includes inter- and intra-molecular di-sulfide bonds made by the oxidation reactions of sulphydryl–disulphide interchange or by sulphydryl reactions) and non-covalent bonds (which includes hydrophobic, hydrogen, ionic, and other weak interactions). PAGE technique is used to characterize the protein components and to differentiate the inter protein interactions which are responsible for the insoluble fractions, and these fractions are high in quantity and varies in particle size. Another main reason reported for the insolubility is the hydrophobic bonding in-between the casein micelles and with some involvement from minor whey proteins. The insoluble fraction continues to increase during the storage

period. There is no contribution of the disulphide bonds (in-between the beta lactoglobulin and kappa casein) in the insolubility of the MPCs. Although, there are several reasons mentioned for insolubility like insolubility reaction on the particle surface, casein micelles interaction, but more scientific data is required to clarify insolubility mechanisms.

2.7.2. Key factors contributing for insolubility of MPCs

According to the (Meena et al. 2017), calcium removal in permeate depends upon the various processing parameters like pH, protein and mineral content of the skim milk, its heating and holding time before the UF, temperature and pH of skim milk during UF, and means of protein concentration (only UF; UF and DF; UF and evaporation; UF, DF and conventional evaporation). The parameters and conditions in spray drying (like inlet and outlet air temperatures, dryer and atomizer type, storage temperature, time and relative humidity, and water temperature during reconstitution), also have important effects on the solubility of MPCs. Hydration time is reduced when hydration is done at 50°C. Moreover, the production method also contributes to the solubility of MPCs. Total solids rise in the UF concentrate either due to diafiltration up to 25% TS or due to evaporation to 31% TS, have significant impact on the solubility reduction of high protein MPCs (especially of MPC with protein content of 80% and higher) (Fang et al., 2011).

2.8. Conclusion

Temperature, pH, UF concentration ratio, DF, and evaporation are the process parameters which affect the solubility of MPCs after production and during storage (Meena et al. 2017). There is a significant effect of high spray drying temperature (155°C and 178°C) on the solubility of MPCs, as compared to the 77°C and 107°C temperatures (Fang et al.

2012). Researchers have tried the different approaches to increase the solubility of MPCs like monovalent ions addition before drying, use of cation exchange technology (Neil et al. 2000), calcium content reduction by chelating (Schuck et al. 2002), high hydrostatic pressure to change the conformation of proteins (Udabage et al. 2012). These approaches have observed a significant increase in the solubility of MPCs. However, most of these studies had determined the solubility affected by various conditions, but those studies have not checked the effect of those conditions 1) on other functional properties of MPCs, and 2) on the relation of solubility and other functionalities. Direct steam injection was not used in the production of MPCs in any of the literature studies. So, to fill these gaps of literature, this study determined the impact of direct steam injection with controlled pH on the functionality of MPCs. This study will help to broaden the use of MPCs in different food processing applications.

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Chapter 3: Direct steam injection processing improves the functionality of milk protein concentrate and non-fat dry milk

3.1. Interpretive summary

The aim of this study was to determine the combined effect of the three pH levels (7, 8, and 9) and two direct steam injection (DSI) temperature levels (85 and 105°C), on the functionality and physiochemical properties of milk protein concentrates, (MPC70 and MPC80) and non-fat dry milk (NFDM). Results from this research indicated an improvement in the solubility, increase in viscosity, decrease in particle size and increase in zeta potential of MPCs due to the DSI treatment of retentate at alkaline pH.

3.2. Abstract

The combined effects of three pH levels (7, 8, and 9), at two direct steam injection (DSI) temperatures (85 and 105°C), on the functionality and physiochemical properties of milk protein concentrates (MPC70 and MPC80), and non-fat dry milk (NFDM or M34) was explored. The control samples of each powder type were produced with no DSI and pH treatment. The samples were evaluated for their proximate composition (total and soluble protein, ζ-Potential, and particle size distribution), and functionality (solubility, heat stability, viscosity, rennet gelation time, emulsification, and foaming capacity and stability). After testing all the samples for functional and physiochemical properties, then from each powder type, two samples of high solubility were selected for the storage study. These samples, along with control, were analyzed after storage at 40°C for 20 days. Means of M70 and M80 samples treated with increased pH at both evaluated temperatures showed an increased solubility, zeta potential, viscosity, and a decreased particle size. Decreased viscosity has been seen in NFDM samples treated with increased pH at both evaluated temperatures. Ash

content showed an increase with increasing pH, with no significant change in protein composition of M70 and M80 samples. Gelation time increased with increasing pH for both temperatures studied. Foaming capacity was not significantly affected with an increase in pH. pH and temperature increase induced the structural changes in samples. Imaged protein microstructures showed decrease in aggregation with an increase in pH in M70 and M80 samples, whereas increase in pH increased aggregation in M34 samples. Depending upon the desired functionality, high pH and DSI treated MPCs could be used to enhance the functionality of fluid systems such as dairy beverages.

Keywords: pH, milk protein concentrate, direct steam injection, protein functionality

3.3. Introduction

Functional properties of MPCs are affected by initial milk composition, salt content and concentration, pH, protein concentration, processing parameters, and extent of protein denaturation (Uluko et al., 2016a). However, the lack of rapid dissolution and decrease in solubility with storage time, particularly in high protein MPCs (Meena et al., 2017), limits their use in many food products, especially products that require rapid rehydration at room temperature (Mimouni et al., 2010). The slow dissolution kinetics leads to poor MPC solubility (Mimouni et al., 2009).

Solubility, in true solution and colloidal dispersion, affects the functionality of components in foods (Mimouni et al., 2010). Solubility is affected by the different issolution steps in water, like wettability, sinkability, and dispersibility (Thomas et al., 2004).

Hydrophobically linked α- and β-caseins are the main components of the insoluble portion of MPC dissolution (Havea, 2006). Casein (CN) micelles colloids are made of nanoclusters (Holt et al., 2003; Holt, 2004). Phosphorylation centers of α_{s1}, α_{s2}- and β-caseins

binds through calcium phosphate ions to the amorphous calcium phosphate core (also known as colloidal calcium phosphate (CCP)) of nanoclusters (Holt et al., 2003; Holt, 2004). Hydrogen bonds (Zadow, 1993), colloidal calcium phosphate (CCP), hydrophobic, and electrostatic interactions (Anema & Li, 2000; Liu & Guo, 2008; Madadlou et al., 2009) are responsible for the structural integrity of CN micelle. The colloidal stability of CN micelle is due to the steric repulsion from the negatively charged outer surface of κ- (κ-CN) and β-caseins (De Kruif et al., 1996; Tuinier et al., 2002). Z-potential, a physical property, is a measure of this charge in MPC dispersion and concentrates (Beliciu et al., 2012). Repulsion of particles in good dispersion is caused by large negative or positive ζ-potential values that prevent aggregation and increases the dispersion stability (Beliciu et al., 2012). As the zeta potential decreases, dispersions can show strong agglomeration and precipitation (0 to +/-3 mV), onset of agglomeration (+5 to -5 mV), threshold of agglomeration (−10 to −15 mV), onset of dispersion (−16 to −30 mV), moderate stability (−31 to −40 mV), fairly good stability (−41 to −60 mV), very good stability (−61 to −80 mV), or extremely good stability (−81 to −100 mV) (Riddick, 1968).

The intramicellar stability decreases with i) breakdown of calcium phosphate ions using calcium-chelating agents or high hydrostatic pressure, or ii) the breakdown hydrophobic bonds using urea or sodium dodecyl sulfate or heating in the presence of ethanol (Vaia et al., 2006). Furthermore, increasing pH causes CN disruption, but this mechanism is poorly understood (Odagiri & Nickerson, 1965; Van, 1992; Vaia et al., 2006). However, Vaia et al. (2006) stated that i) increased pH decreases the ionic calcium and phosphate levels, which increases the solvent quality and decreases the intra CN micelle hydrophobic

interaction, and ii) CN micelle break down occurs into individual nanoclusters because of an elevated negative charge, with no disruption of calcium phosphate ion pairing.

Heat coagulation time (HCT) of reconstituted MPCs is determined to ensure uniformity and quality of their heat-induced stabilization in ultra-high temperature processing or retort stabilization. Steric destabilization of CN micelle occurs due to mineral equilibrium change from serum to colloidal phase at HCT temperature. HCT of MPCs depends on calcium ion concentration, pH, and protein concentration. At pH > 6.7, a decrease in HCT of MPCs suspension is due to enhanced Ca ion activity (decreased serum mineral content, mainly soluble calcium) along with κ-casein dissociation due to heat treatment (Crowley et al., 2014). Calcium chelating salt or phosphate addition decreases the Ca-ion activity and enhances the buffering capacity and serum mineral content, which leads to an increase in the HCT (McSweeney & Fox, 2009).

In foaming, proteins act as a surfactant and decrease the surface tension between gas and water (Huppertz, 2010). So, the proteins play an important role in forming the desirable air-water dispersion in products like ice cream, cakes, whipped toppings, etc. (Uluko et al. 2016). Heat treatment, pH, and ionic environment affect the foaming properties of milk proteins (Ward et al., 1997; Hagolle et al., 2000; Zhang & Goff, 2004). As β-caseins have unordered structure and high mean residue hydrophobicity, which increase their surface-activity, when compared to the other caseins like κ-, α_{s1}-, and α_{s2}- caseins. Thus, β-caseins adsorb at the air interface rapidly (McSweeney & Fox, 2009). Even though both β-lactoglobulin and α- lactoalbumin have a significant amount of α- helix, β- sheet and intramolecular disulfide bonds (Fox & Mcsweeney, 2015). But they adsorb at the air interface slowly because their hydrophobic residues are buried within the molecules

(Damodaran & Paraf, 2017). Foaming is also improved when CN micelle dissociates due to the removal of ionic Ca (Zhang & Goff, 2004). At pH 7 and 8, foaming ability improvement is presumably due to the dephosphorylation of caseins (Zhang et al., 2004).

The viscosity of the milk concentrate is directly proportional to the volume fraction (or voluminosity) of the milk proteins (Anema et al., 2004). The voluminosity of milk proteins depends upon pH (Creamer & Anema, 1993), temperature (Snoeren et al., 1984), casein genetic variants (Creamer & Anema, 1993), and colloidal calcium phosphate (CCP) levels (Creamer & Anema, 1993). Aggregation of milk proteins increases the viscosity of skim milk (Langley & Temple, 1985). Dissociation of CN micelle reduces the viscosity of milk likely due to a decrease in the voluminosity of CN micelle (Anema, 2008).

MPCs are easily dissolved at higher temperatures during the cheese-making process. So, the insolubility of MPCs at 60°C for 5 minutes doesn't have a significant effect on the cheese-making process, because it has been added before pasteurization (Martin et al., 2010). However, rennet gelation property significantly influences the cheese-making process, which is related to the calcium activity, the insoluble calcium: phosphate ratio, and micellar integrity (Ferrer et al., 2008). Therefore, if there is any change in calcium activity of MPC, it will affect the cheese making process.

In recent years, there have been various studies to improve the solubility of milk protein concentrate powders. Bhaskar et al. (2003) patented different methods for calcium removal from UF retentate, including the cation exchanger method, addition of calcium chelators, and acidification of skim milk. These methods lead to enhancement in the solubility of MPC70. Udabage et al. (2012) observed an increase in the MPC solubility when UF retentate or skim milk was treated with 200 Mpa at 40°C using high-pressure processing.

The proposed mechanisms for the increase in solubility were i) conformational change in the non-micellar casein and ii) high mineral-salt content in milk. Augustin et al. (2012) reported that high shear treatments to UF retentate including homogenization (300/100 bar), microfluidization (800 bar), and ultrasonication (24kHz, 160 ml/min @ 600 W), lead to increase in the nitrogen solubility index of MPC 82 from 70.14% (control) to 74.46, 89.52, and 74.69 % respectively. The physical interventions were responsible for the increase in solubility. Sun et al. (2014) have used the power ultrasound pre-treatment (20 kHz, 12.50 ± 0.31 W, and 50% amplitude) to enhance the functionality of MPC 80, such as solubility, emulsification, and gelation, and correlated these changes to increase in particle size and surface hydrophobicity. The addition of NaCl during the diafiltration results in an increase in the solubility of MPC80, presumably due to modification in the hydrophobic sites, reduced formation of disulfide bonds, and decrease in particle size (Mao et al., 2012). Sikand et al. (2013) also observed an increase in solubility and a decrease in turbidity of MPC 80 with the addition of NaCl and KCl. Banach et al. (2013) stated an increase in solubility of MPC80 using controlled enzymatic hydrolysis of retentate with digestive enzymes; i) chymotrypsin, trypsin, and pepsin, and ii) cysteine protease – papain.

High temperatures and short time combinations are used in the direct steam injection (DSI) process (Lewis & Heppell, 2000). The functionality of soy flours, concentrates, and isolates were improved using the DSI process (Wang & Johnson, 2001). DSI treated soy protein concentrate restored solubility approximately to that of the native protein. The solubility of soy concentrate, and isolate was improved with longer time of DSI processing; however, a darker color was observed in soy isolate due to the production of Maillard

reaction products. The authors stated that the functionality of DSI treated soy concentrate is improved due to more than one biochemical mechanism (Wang & Johnson, 2001).

Ganjyal et al. (2011) patented a DSI processing method that was used to improve the functionality and/or nutritional properties of protein blends. The proposed mechanism for improved functionality was unfolding of protein structure due to the combined effect of pH adjustment and thermal shock, and then conformational rearrangement during cooling. Moreover, DSI expected to produce the cross-linked hybrid proteins from two or more protein sources due to change in disulfide bonds (SS) and sulfhydryl groups. However, more analysis is required to support this hypothesis.

Pietrysiak et al. (2018) observed an increase in solubility, foam stability, emulsifying activity index, and oil holding capacity of the combination of rice and pea proteins using DSI at temperature 107°C and pH 9, with no change in the amino acid profile of the proteins. This suggests that the primary structure of proteins is not changed. Therefore, it was concluded that only secondary, tertiary, and quaternary protein structures are affected by DSI treatment, with no effect on the primary structure of the protein. Thus, the objective of this study was to determine the combined effect of three pH levels (7,8, and 9), at two DSI temperatures (85 and 105°C) on the functionality and physiochemical properties of MPC70, MPC80, and non-fat dry milk (NFDM). pH and temperatures were selected based on these reasons: i) more effective protein conformational change occurs at high pH (Fox et al. 2015)and ii) more whey protein denaturation happens very quickly at the temperature above 80°C (Donovan et al. 1987). MPC 70, and 80 were selected for production and DSI treatment, because these are commonly produced MPCs. NFDM was manufactured and treated for the comparison purposes.

3.4. Materials and methods

3.4.1. Materials

Pasteurized skim milk was received from Terry's Dairy Inc. (Colville, WA). Analytical grade chemicals were purchased Sigma-Aldrich Chemical Ltd. (St. Louis, MO), and Thermo Fisher Scientific, Waltham, MA. All solutions were made using deionized water.

3.4.2. Production of MPC70, MPC80, and NFDM

All protein powders were made in duplicate. MPCs and NFDM were manufactured using 10-KDa cut-off, spiral-wound, polyethersulfone ultrafiltration (UF) membranes (Koch Membrane systems, Inc. Wilmington, MA), and spiral-wound, polyamide reverse osmosis (RO) membranes (Hydranautics-Nitto Group Company, Oceanside, CA), respectively. The temperature at the beginning of UF and RO, was maintained at 5 ± 1.5°C (mean ± SD), and then allowed to increase up to 20 ± 1.5°C during UF. Water used for diafiltration had pH 8 ± 0.1. For MPC production, high protein content ultrafiltration retentate was made and standardized with permeate to get the desired protein content in MPCs.

For each powder type, the retentate was divided into three batches with every batch containing two samples, and the pH was adjusted to 7.00 ± 0.01, 8.00 ± 0.01, and 9.00 ± 0.01 for the three batches respectively, using the 0.1 M NaOH. The two samples from each batch were treated at 85 ± 2°C and 105 ± 2°C respectively using DSI (EZ Heater H2010, Hydro - Thermal Corporation, Waukesha, WI, U.S.A) for 33 ± 1 s at 5 L/min flow rate. However, we found aggregation in the 9-pH treated M34 sample after DSI injection at 105 C. Therefore, we removed the 9-pH treated DSI injected at 105 C M34 sample from our analyses. All the samples were kept under refrigeration conditions overnight and then neutralized to a pH of 7.0 using 1 M HCl. All samples were dried using a spray dryer (Model Lab 1, Anhydro Inc.,

Soeberg, Denmark) operated with the inlet and outlet air temperatures of 200 ± 2 and 90 ± 2°C, respectively, using a 2-fluid nozzle with compressed air. The feed flow rate was used to control the outlet air temperature. After cooling to ambient temperature (20 ± 2°C), the spray-dried powders were stored in 14-16 oz silver metallized SUP bags (Pacific Bag, Inc., Woodinville, WA), and, immediately, stored at -18°C, until further analysis. The control samples of MPCs and NFDM were made with no DSI and pH treatment. We assumed that short time temperature treatment (85 ± 2°C and 105 ± 2°C for 33 ± 1 s) at 7, 8, and 9 pH would not produce the lysinoalanine (LAL) and racemize the amino acid residues in casein, thus will not affect the digestibility of casein, as reported by Friedman et al. (1981). After testing all the samples for functional and physiochemical properties, then two samples with high solubility, smaller particle size, and high zeta potential were selected from each powder type and stored for 20 days at 40°C, with the control sample. After storage of 20 days at 40°C, the solubility and microstructure of powders was determined.

3.4.3. Experimental Design

A randomized block factorial experimental design, with a total of 30 samples, including three levels of protein content, three pH levels, and two DSI temperature levels, and two replications was utilized (Table 3.4.3.1, and 3.4.3.2).

Table 3.4.3.1. Experimental design for MPC and NFDM production

Protein	**pH**	**Temperature (°C)**	**Total Samples (in duplicate)**
34% (NFDM)	7	85	(17*2) + 6[a] = 40
70% (MPC)	8	105	
80% (MPC)	9		

[a] Control of MPCs and NFDM was made by giving no DSI and pH treatment to the powders. We found aggregation in the 9-pH treated M34 sample after DSI injection at 105 C. Therefore, we removed the 9-pH treated DSI injected at 105 C M34 sample from our sampling.

Table 3.4.3.2. Samples labeling for MPC and NFDM production

	Different powder produced		
	NFDM	**MPC70**	**MPC80**
Treatments	M34N85	M70N85	M80N85
	M34H85	M70H85	M80H85
	M34HH85	M70HH85	M80HH85
	M34N105	M70N105	M80N105
	M34H105	M70H105	M80H105
		M70HH105	M80HH105
	M34	M70	M80

M34, M70, and M80 represent the NFDM, MPC70, and MPC80, respectively. N, H, and HH represent samples treated at 7, 8, and 9 pH, respectively. 85 and 105 represent samples treated at 85 and 105 C in DSI treatment, respectively.

3.4.4. Compositional Analyses

Moisture and ash contents was evaluated using the standard evaluation methods for dairy powders (Wehr & Frank, 2004). Mojonnier method (Wehr & Frank, 2004) was used to determine the fat content of dispersed powder (5%, wt/wt) at 60°C for 5 minutes, and the calculation was done accordingly (Rupp et al., 2018). The Dumas method (FP-528, Leco Corporation, St. Joseph, MI, U.S.A) was used to determine the protein content.

Sodium dodecyl sulfate-PAGE was used for the protein composition analysis in both soluble and total fractions of dispersed powder samples under reducing and non-reducing conditions (Fang et al., 2012), with slight modifications. 0.5 % (w/vol.) dispersion of powdered samples was stirred at 800 rpm at 50ºC for 30 minutes. 10 µL of sample, before centrifugation, was taken to get total protein fractions. 10 µL of supernatant was taken after

centrifugation at 5000 rpm for 10 min to get soluble protein fractions. Samples were vortexed with the addition of 10 μL of the Lamile sample buffer and heated for 5 minutes at 95ºC in a water bath (Eshpari et al., 2017). After cooling to room temperature, at 22ºC, the samples were reduced using 5 μL of ß- mercaptoethanol and vortexed for 30 seconds. The reduced and non-reduced samples were loaded into the precast gel (4 - 15% precast polyacrylamide gel, Bio-Rad Laboratories) and ran at a constant voltage of 200 V for 55 minutes. Coomassie Brilliant Blue was used for staining of the gels. Novex Sharp Pre-Stained Protein Standards (Invitrogen, Waltham, MA, USA) were used as molecular weight markers.

ζ-Potential and intensity-weighted average hydrodynamic diameter (d_h or d_{err}) of samples were determined by dynamic laser scattering technique using Brookhaven NanoBrook ZetaPALS with analyzing parameters taken from the literature (Hunter, 1981; Langman & Moberly, 2018). The powdered sample was dispersed in deionized water at 3% (w/vol.) at 20ºC for 1 h and was left overnight at 4ºC. The sample was heated to 50ºC for 5 min to restore CN micelle integrity. Just before analysis, the sample was diluted 100 folds, vortexed for 10s, and pipetted into single-use cuvettes. ζ-Potential and particle size analysis tests were started after sample temperature equilibration at 25ºC for 30 and 60s, respectively. Each measurement consisted of three subsequent individual runs, including 30 cycles per run, with an intracycle delay of 1 and 0s for ζ-Potential and particle size analysis, respectively. Each measurement was completed in less than 10 min to keep the integrity of the CN micelle by limiting the mineral equilibration between the CN micelles and aqueous environment (Luo et al., 2015). BIC software (Brookhaven Instruments Corp., Holtsville, NY, USA) was used for data collection and analysis. The dust filter cut-off parameter was set at 40, as

suggested by the manufacturer (Beliciu & Moraru, 2009). Measurements were performed in duplicate.

3.4.5. Functionality Tests

3.4.5.1. Solubility

MPC80, MPC70, and NFDM powder samples were dispersed at 4.2%, 4.8%, and 11.11% (w/vol.), respectively, at 20ºC for 1 hour at 600 rpm, to get the protein profile similar to that of skim milk (Anema et al., 2006; Martin et al., 2010). Samples left overnight at 4ºC, to ensure complete rehydration. Solubility was determined using the method reported by Rupp et al. (2018) with slight modifications. 25 ml of the dispersion was centrifuged at 2,460 × g for 10min at 21ºC. After decanting the supernatant, the sample was diluted with 25 ml of deionized water and re-centrifuged. Supernatant decanted, and the pellet was vortexed in 5 ml of deionized water. TS content of pellet and original solution was compared using the following equation to get insolubility (%) and solubility (%):

$$Insolubility\ (\%) = 100 - solubility$$

$$= \left(\frac{Total\ solids\ content\ pellet}{Total\ solids\ content\ original\ solution}\right) * 100$$

3.4.5.2. Foaming

Sample dispersions were prepared as detailed in the solubility test method. For the foaming test, 10 mL of dispersed sample was homogenized for 5 minutes in a 50 ml graduated cylinder using a homogenizer (Ultra-Turrax TP 18/10S1, Janke & Kunkel, Saufen, Germany) at its highest setting. Foaming capacity and stability were measured using the method reported by Shilpashree et al. (2015).

3.4.5.3. Viscosity

Samples dispersions were prepared, as mentioned in the solubility test method. Flow profiles were evaluated with a rheometer (MCR 302 Anton Paar; Gratz, Austria) equipped with a cup (diameter 22.199 mm) and bob (diameter 21.006 mm, and length 51.907 mm) attachment. The cup and bob attachment had an annular gap of 0.158 mm. In pre-test procedure, the sample was loaded using the disposable transfer pipette and equilibrated at 25 °C for 60 s, and after that pre-sheared at 10 s^{-1} for 20 s and allowed to rest for 20 s. Shear-dependent behavior was evaluated by collecting the shear rate sweeps (from 0.01 to 100s^{-1}) at 25ºC. Flow profile data were collected from the Rheocompass software (Anton Paar, Graz, Austria).

3.4.5.4. Heat stability

Heat stability was determined using the method reported by Crowley et al. (2014) and Davies & White, (1966) with slight modifications. The powdered sample was dispersed in deionized water at 3% (w/vol.) at 20ºC for 1 h and was left overnight at 4ºC. 3 mL of dispersion was held at 140ºC in the 10 mm diameter glass tubes in uncovered oil bath (Oil Bath DL30-W15/B 25, Thermo Fisher Scientific, NH, USA) until coagulation was visually observed. Coagulation time was measured and recorded.

3.4.5.5. Rennet Gelation

Same concentrations of samples, as described in solubility test method, were made in duplicate in 2% sodium azide solution at 60ºC for 5 min at 600 rpm, to ensure complete solubilization (Eshpari et al., 2017; Martin et al., 2010). Samples were supplemented with 2 mM $CaCl_2$ after cooling to room temperature, reconstituted for 3 h at 300 rpm, and kept overnight at 4ºC, to ensure complete rehydration. Samples were equilibrated at 30ºC for 5

min at 300 rpm, and 0.0315 international milk clotting units (IMCU) concentration of rennet (CHY-MAX® Extra, CHR Hansen, Inc., Hoersholm, Denmark) per ml, was added. Samples were vortexed for 10 s, before transferring to the Anton Paar MCR 302 rheometer (Anton Paar; Gratz, Austria) in the cup (diameter 22.199 mm) and bob (diameter 21.006 mm, and length 51.907 mm) attachment. The cup and bob attachment had an annular gap of 0.158 mm. In pre-test procedure, the sample was loaded using the disposable transfer pipette and equilibrated at 30 °C for 60 s, and after that pre-sheared at 10 s^{-1} for 20 s and allowed to rest for 20 s. During the test, the sample was set for structure recovery for 1 minute. Gelation time was taken as the crossover point of storage and loss modulus using amplitude sweep from 0.01 to 100%, indicating a shift from viscoelastic fluid to viscoelastic solid behavior, at 30ºC with a frequency of 1 rad/s. Starting of gel formation is indicated by increase in storage modulus (Eshpari et al., 2017).

3.4.6. Microstructure

The microstructure of the rehydrated milk powder was investigated by the scanning electron microscopy (SEM). Samples at 5% (w/vol.) were stirred at 20ºC on a stirring plate at 800 rpm for long-term rehydration (80 min) (Mimouni et al., 2010).

Suspension was chemically fixed for 15 minutes at room temperature using the 3% glutaraldehyde in 100 mM phosphate buffer (pH 7). After this, samples were microwave fixed at 250 W for 1 minute at 28º C. Sample was rested for 5 min at 20ºC and centrifuged at 2000 g (Mini Centrifuge, Benchmark Scientific, Sayreville, NJ, USA) for 5 min to get a pellet. The pellet was rinsed with deionized water for 3 times with 10 minutes time for each rinse. Graded ethanol series: 50, 70, 90, 95 (2 times), and 100% (3 times) with elapse time of 5 minutes in between each solution, was used to dehydrate the pellet. After this, pellets were

chemically dried using Hexamethyldisilazane (HMDS) for 5 minutes, kept in a fresh solution of HMDS for overnight, and transferred to vacuum desiccator. The sample was mounted on microscopy stubs using a double-sided carbon adhesive tape and coated with platinum to a 3 nm thickness for 2 minutes. The sample was examined using field emission SEM (Model Quanta 200F, FEI Company, USA) at an accelerating voltage of 20 kV. We have obtained images at 25K, 50K, and 100K x as most of the samples yielded good resolution images at these magnification levels.

3.4.7. Statistical analysis

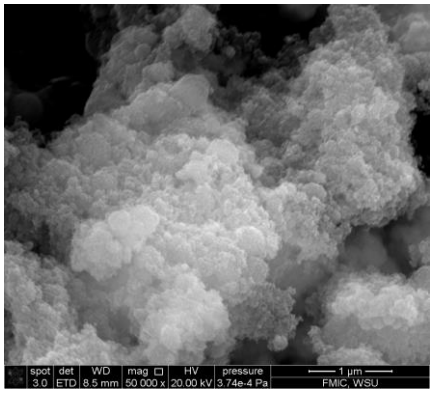
A statistical software (SAS 9.1; Cary, NC) was used to analyze the randomized block design for each MPC type with MPC treatment as a treatment factor and duplicate as a block. Compositional and functionality data were analyzed by ANOVA, followed by Tukey's HSD to identify significant differences at $P < 0.05$. Storage study was analyzed using split-plot factorial with whole plot as randomized complete block design. Whole plot had MPCs treatments as a treatment factor and duplicate as a block. Split-plot had storage time as a split-plot factor.

3.5. Results and discussion

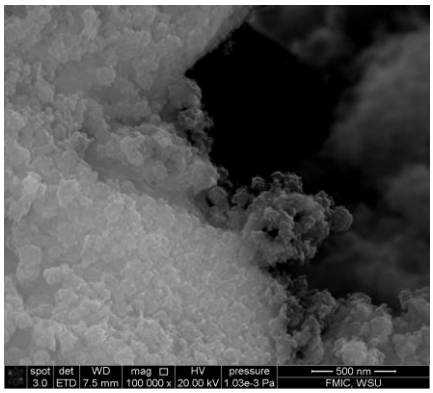
3.5.1. Microstructure

The microscopy images or micrographs (Figure 3.5.1 and 3.5.2) showed that increased pH in M70 and M80 samples decreased the aggregation of casein micelles, however increased pH increased the aggregation in M34 samples (Figure 2.5.3). Glutaraldehyde has crosslinked the proteins and microwave fixation increase the diffusion rate, and which enhanced the absorption of glutaraldehyde and cross linkage of proteins. In micrographs, cluster of particles (including any glutaraldehyde polymers between casein

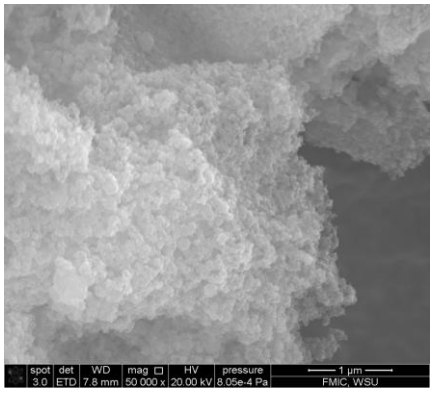
micelles) covered with the platinum has been observed. After long period of rehydration (80 minutes) the order of shorts bridges and direct intermicellar contacts (Mimouni et al., 2010) in powder particles is 9 pH < 8 pH < 7 pH < control samples in both M70 and M80 samples, as observed in micrographs, whereas the opposite effect has been observed in M34 samples. These short bridges and intermicellar contacts prevent the dispersion of individual micelles. Decrease in the hydrodynamic diameter and increase in zeta potential with increased pH at both temperatures of M70 and M80 samples also support the easy dispersion of casein micelles. As stated by Mimouni et al., 2010, glutaraldehyde does not change the size and characteristics of the native interactions between casein micelles, however, it might change the internal structure of casein micelles. Washing step after fixation should have separated out the casein micelles which were held together due to surface tension (Mimouni et al., 2010).



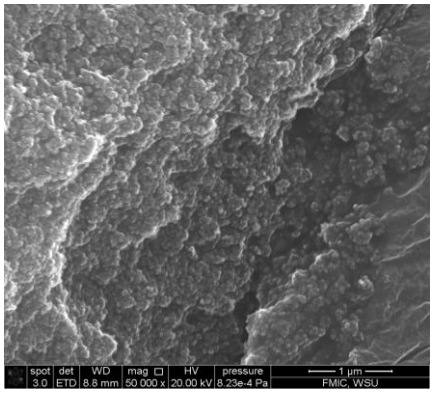
M70



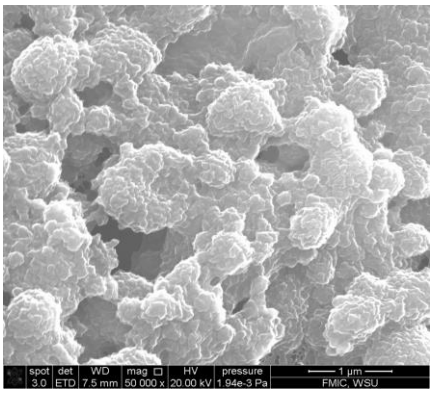
M70N85



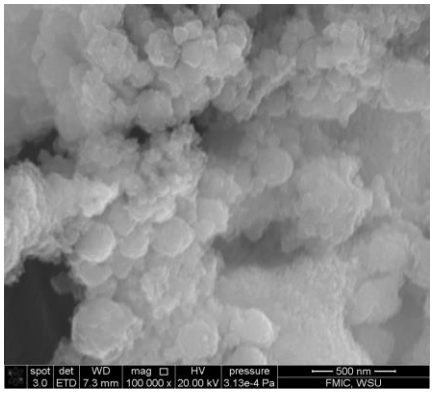
M70H85



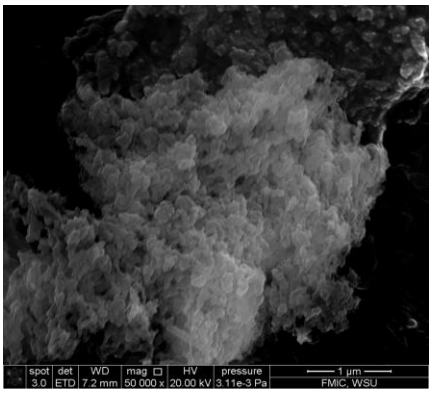
M70HH85



M70N105

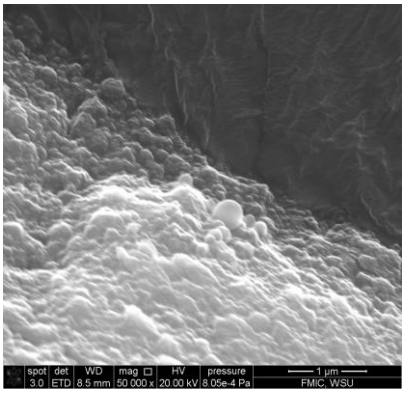


M70H105

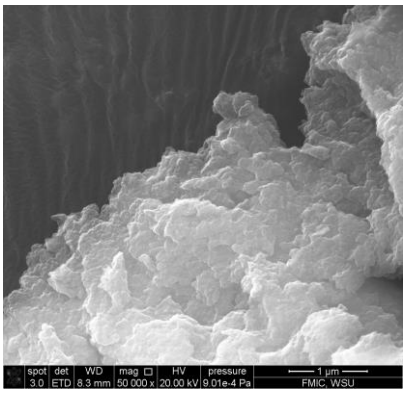


M70HH105

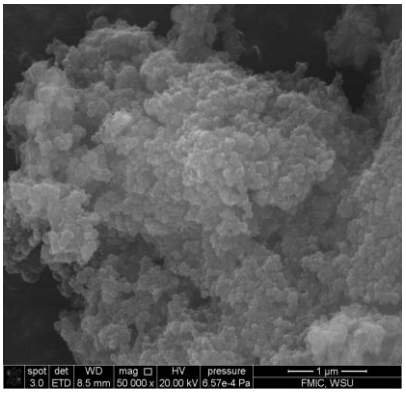
Figure 3.5.1. Microscopy images of different treatments of M70 samples



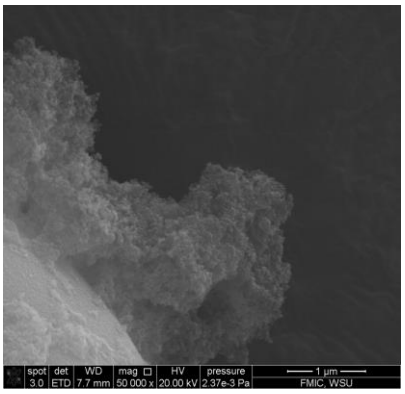
M80



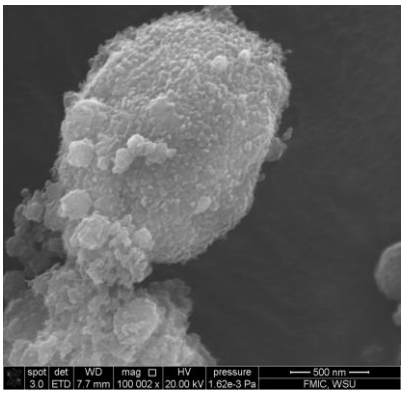
M80N85



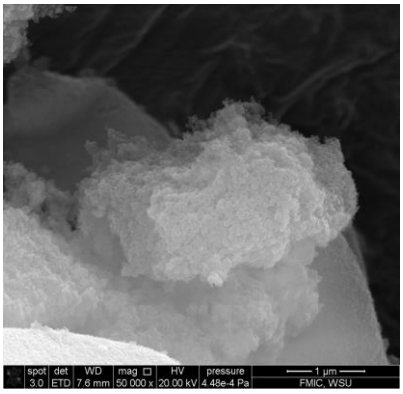
M80H85



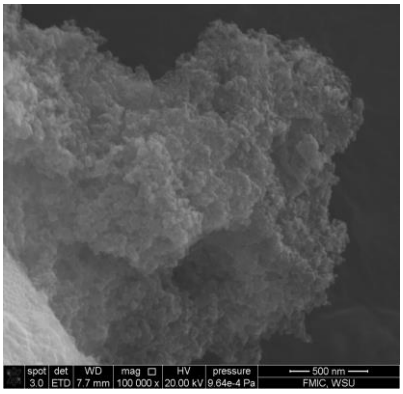
M80HH85



M80N105

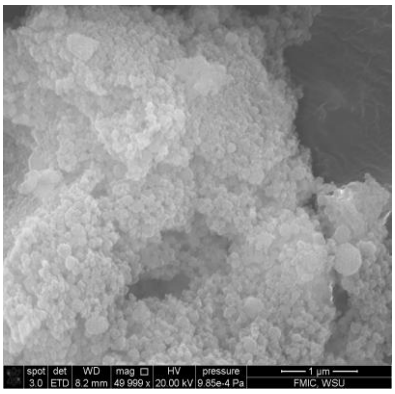


M80H105

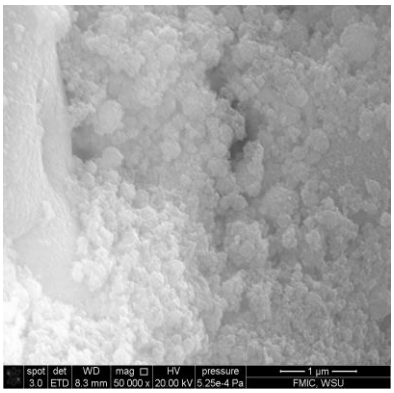


M80HH105

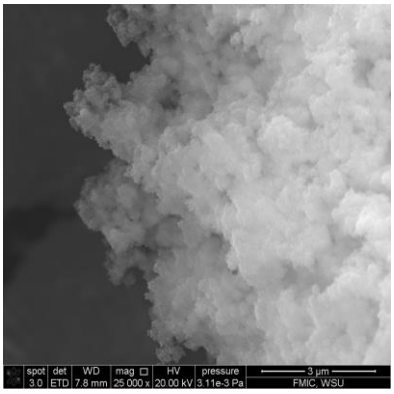
Figure 3.5.2. Microscopy images of different treatments of M80 samples



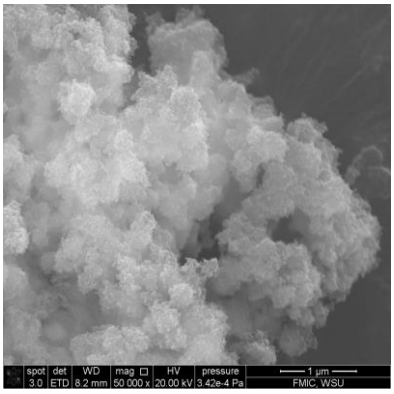
M34



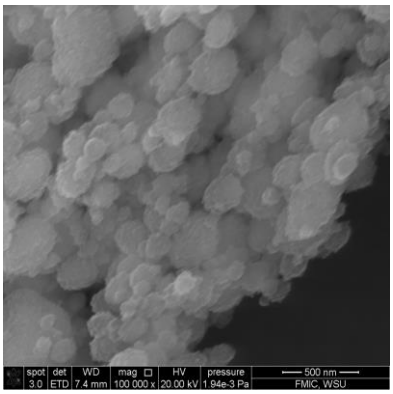
M34N85



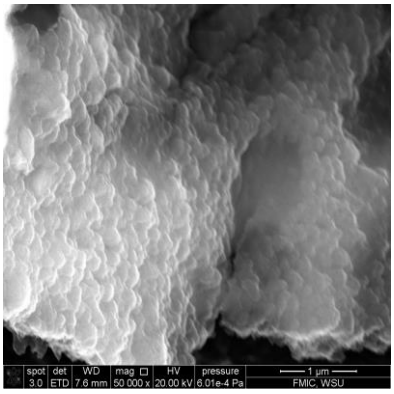
M34H85



M34HH85



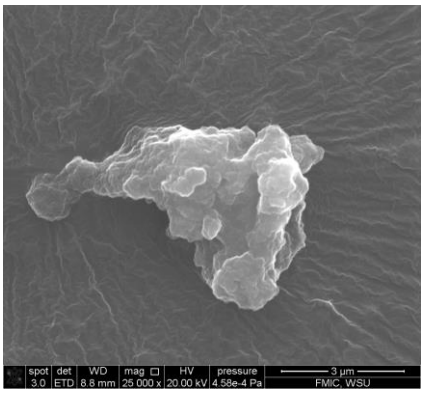
M34N105



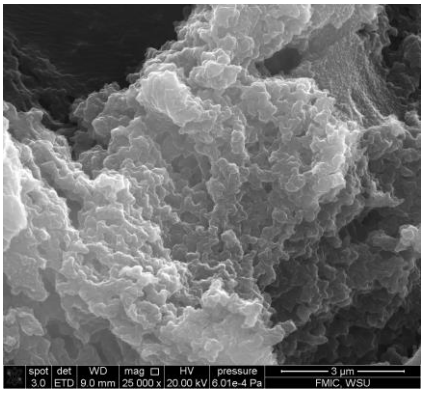
M34H105

Figure 3.5.3. Microscopy images of different treatments of M34 samples

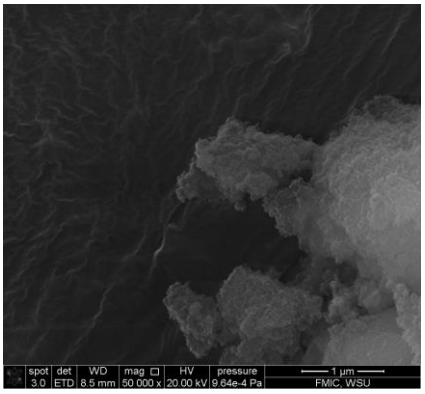
After 20 days of storage time, microscopy images of 9 pH treated samples of M70 and M80 (Figure 3.5.4 and 3.5.5) showed a decrease in the aggregation of casein micelles as compared to control samples. Whereas microscopy images of 7 pH treated samples and control samples of M34 (Figure 3.5.6) showed an aggregation of casein micelles.



M70

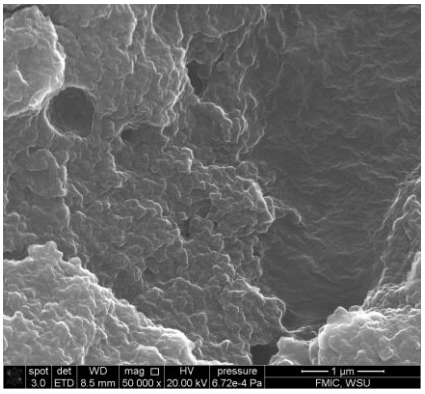


M70HH85

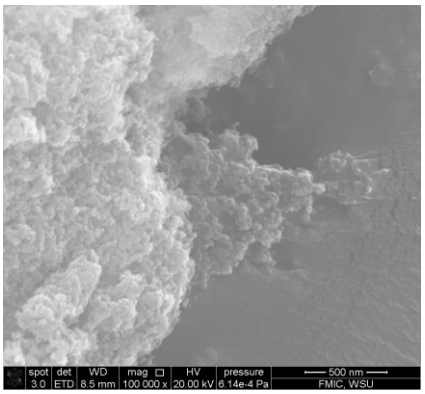


M70HH105

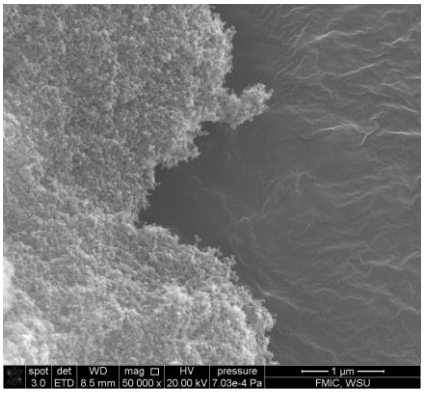
Figure 3.5.4. Micrographs of different treatments of M70 samples after storage time



M80

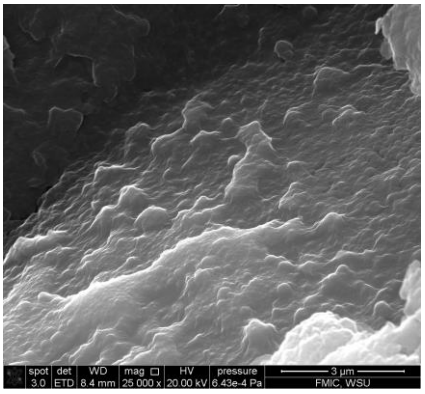


M80HH85

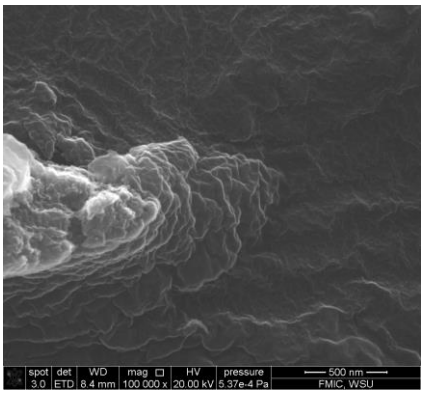


M80HH105

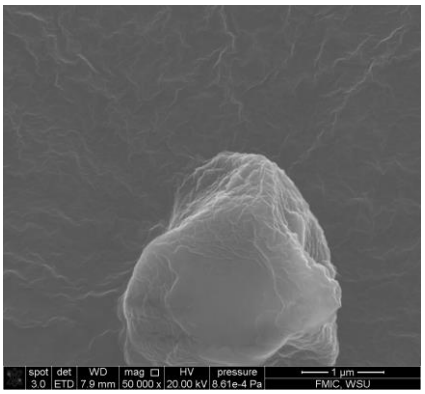
Figure 3.5.5. Micrographs of different treatments of M80 samples after storage time.



M34



M34N85



M34N105

Figure 3.5.6. Micrographs of different treatments of M34 samples after storage time

3.5.2. Composition and physical properties

The results of the proximate composition analysis indicate that there was no significant difference (P>0.05) in fat, protein, and moisture content of M70 and M80 samples. However, ash content increased (P<0.05) with increase in pH at both temperatures in M70, M80, and M34 samples (Table 3.5.2.1), probably because of NaCl salt formation during the neutralization of NaOH with HCl. M34 samples were not significantly different (P>0.05) in fat, and moisture content. Whereas protein was significantly different in M34 samples (P<0.05). The pooled protein content of NFDM, MPC70, and MPC80 was 34.6%, 70.9%, and 80.9%, on DMB basis.

Table 3.5.2.1. Mean ash content (%) of different treatments of nonfat dry milk (M34), MPC70 (M70), and MPC80 (M80) samples. Values with the same superscript within a column were not significantly different (P > 0.05).

Treatment	pH	Heating (°C)	Ash Content (%)		
			M34	M70	M80
Control	-	-	7.81^{d}	6.76^{c}	6.57^{c}
N85	7	85	8.13^{c}	6.95^{bc}	6.74^{bc}
H85	8	85	8.43^{b}	7.29^{ab}	6.97^{b}
HH85	9	85	8.72^{a}	7.69^{a}	7.43^{a}
N105	7	105	8.11^{c}	6.86^{bc}	6.76^{bc}
H105	8	105	8.46^{b}	7.22^{b}	7.06^{b}
HH105	9	105	-[1]	7.69^{a}	7.47^{a}
Pooled SEM			0.039	0.075	0.061

[1]No sample, treated milk coagulated before spray drying.

In our study, the zeta potential of CN micelle increased significantly (P<0.05) (Table 3.5.2.2) with an increase in pH at both temperatures in M70, whereas increased pH had not significantly increased the zeta potential of M34 samples. Means of zeta potential of CN micelle increased (Table 3.5.2.2) with an increase in pH at both temperatures in M80 samples, even though there was no significant difference (P>0.05) in the means of zeta potential of CN micelle of M80 sample treatments. This was because of the enhanced electrostatic repulsion of dissociated CN micelle.

Table 3.5.2.2. Zeta potential (mV) (mean ± SEM) of different treatments of M34, M70, and M80 samples. Values with the same superscript within a column are not significantly different ($P > 0.05$).

Treatment	pH	Heating (°C)	Zeta Potential (mV)	
			M70	M80
Control	-	-	-41[a]	-41[a]
N85	7	85	-32[ab]	-32[a]
H85	8	85	-35[ab]	-36[a]
HH85	9	85	-39[ab]	-39[a]
N105	7	105	-31[b]	-32[a]
H105	8	105	-32[ab]	-34[a]
HH105	9	105	-41[a]	-40[a]
Pooled SEM			1.593	1.839

[1]No sample, treated milk coagulated before spray drying.

There was no significant difference ($P>0.05$) was detected in CN micelle size of M70 and M80 sample treatments. But we found a decrease in the means of CN micelle size with an increase in pH of M70, and M80 sample treatments (Table 3.5.2.3). Decrease in size indicated the disruption of CN micelle, as reported in the sonodisruption study published by Madadlou et al. (2009). Increased pH resulted in a significant ($P<0.05$) increase in the CN micelle size of M34 samples (Table 3.5.2.3). Aggregation tendency of caseins is inversely proportional to the dissociation of individual CN (Dumpler et al., 2017). So, in our study, we can reason that i) the increase in pH caused the weakening of structure of CN micelle and enhancing of the zeta potential, and ii) high-pressure DSI was responsible for the disruption and decrease in size of CN micelle. In our study, samples with smaller particle size and high zeta potential led to good dispersibility and low rate of sedimentation, similar to that reported in the literature (Blagovidova & Tentsova, 1961; Fang et al., 2011; Kumar & Dixit, 2017).

Table 3.5.2.3. Hydrodynamic diameter (d_h or d_{err}) (mean ± SEM) of different treatments of M34, M70, and M80 samples. Values with the same superscript within a column are not significantly different ($P > 0.05$).

Treatment	pH	Heating (°C)	Hydrodynamic diameter (dh or derr)		
			M34	M70	M80
Control	-	-	106[ab]	104[a]	109[a]
N85	7	85	90[b]	106[a]	146[a]
H85	8	85	92[b]	94[a]	101[a]
HH85	9	85	130[a]	75[a]	83[a]
N105	7	105	89[b]	132[a]	134[a]
H105	8	105	103[ab]	102[a]	115[a]
HH105	9	105	-[1]	73[a]	83[a]
Pooled SEM			5.924	10.665	13.771

[1]No sample, treated milk coagulated before spray drying.

Vaia et al. (2006) stated that the decreased concentration of ionic calcium and phosphate, with an increase in pH, stabilizes the CCPs. Hydrophobic interactions are not significantly affected by the pH (Anema, 1998; Madadlou et al., 2009). This indicates that electrostatic interactions have a high impact on the pH-dependent behavior of CN micelle (Madadlou et al., 2009). Electrostatic repulsion (indicated by zeta potential) increases at high pH due to the shifting of phosphoryl residues from single to double negatively charged units (Horne, 1998), which enhances dispersion stability (Liu & Guo, 2008). Cohesive interactions between hydrophobic regions of CN micelle are inversely proportional to the solvent quality of milk serum (Vaia et al., 2006; Madadlou et al., 2009). A decrease in the ionic

concentration of calcium and phosphate in the CN micelle, with an increase in pH, improves the solvent quality (Vaia et al., 2006; Madadlou et al., 2009). Madadlou et al. (2009) proposed that the addition of hydroxyl ions from sodium hydroxide, as in our study, increases the electrical conductivity, and dielectric constant of the medium, which improves the solvent quality in the medium. This may result in the formation of more hydrogen bonds between serum and colloidal phases, with the breakdown of some hydrogen and hydrophobic bonds in casein chains, which leads to the weakening of the structure of the CN micelle. Yet attractive forces remain sufficient to hold the integrity of CN micelle.

No compositional changes were observed in the total and soluble fractions of protein samples of M34, M70, and M80 as a result of the various treatments studied, under reducing and non-reducing conditions.

3.5.3. Functional properties of MPC

Alkaline pH significantly decreased ($P<0.05$) the heat stability at both temperatures in M70, and M80 samples (Table 3.5.3.1) as compared to the control sample. Whereas alkaline pH resulted in a significant increase in the heat stability of M34 samples (Table 3.5.3.1). Decrease in heat stability indicated that there was probably a decrease in Ca ion activity (Barone et al., 2021).

Table 3.5.3.1. Heat stability (minutes) (mean ± SEM) of different treatments of M34, M70, and M80 samples. Values with the same superscript within a column are not significantly different ($P > 0.05$).

Treatment	pH	Heating (°C)	Heat stability (min)		
			M34	M70	M80
Control	-	-	26^{b}	29^{a}	36^{a}
N85	7	85	28^{ab}	25^{ab}	18^{b}
H85	8	85	31^{ab}	24^{ab}	23^{ab}
HH85	9	85	35^{a}	24^{ab}	28^{ab}
N105	7	105	28^{b}	16^{c}	18^{b}
H105	8	105	30^{ab}	21^{bc}	24^{ab}
HH105	9	105	-[1]	24^{ab}	27^{ab}
Pooled SEM			1.084	1.099	2.897

[1]No sample, treated milk coagulated before spray drying.

There was no significant difference (P>0.05) in foaming capacity and stability of M34, M70, and M80 samples.

The flow behavior of the samples was checked using the Herschel Bulkley model. Viscosity of M80 samples increased with an increase in pH at both temperatures (Table 3.5.3.2), however, control even though there was no significant difference (P > 0.05). We observed a significant (P<0.05) increase in the viscosity of M70 samples with an increase in pH at both temperatures (Table 3.5.3.2). M34 samples showed significant (P<0.05) decrease in the viscosity with an increase in pH at both temperatures. The decrease in hydrodynamic radius of samples with the uplift of pH indicated the dissociation of CN micelle in M70 and

M80, and free released casein resulted in an increase in the water holding capacity (Barone et al., 2021). This increase in water holding capacity enhanced the viscosity of M70 and M80 samples. We observed an increase in viscosity with an increase in pH at both temperatures in M34 samples (Table 3.5.3.2).

Table 3.5.3.2. Viscosity (mPa.s) (mean ± SEM) of different treatments of M34, M70, and M80 samples calculated using shear rate sweeps (from 0.01 to 100s^{-1}) at 25ºC. Values with the same superscript within a column are not significantly different ($P > 0.05$).

Treatment	pH	Heating (°C)	Viscosity (mPa.s) (yield stress ~ 0, n = 1)		
			M34	M70	M80
Control	-	-	1.6^{b}	1.6^{a}	1.55^{a}
N85	7	85	1.8^{ab}	1.4^{b}	1.4^{a}
H85	8	85	1.85^{ab}	1.4^{b}	1.35^{a}
HH85	9	85	2.15^{a}	1.45^{ab}	1.45^{a}
N105	7	105	1.8^{ab}	1.45^{ab}	1.45^{a}
H105	8	105	2^{a}	1.4^{b}	1.4^{a}
HH105	9	105	$-^{1}$	1.55^{ab}	1.5^{a}
Pooled SEM			0.06055	0.02673	0.0378

[1]No sample, treated milk coagulated before spray drying.

With an increase in pH, there was significant increase ($P<0.05$) in the solubility of M80 samples (Table 3.5.3.3). We found that means of M70 samples showed an increase solubility with increase in pH at both temperatures, however there was no significant difference ($P>0.05$) detected in solubility of M70 sample treatments (Table 3.5.3.3). Reduction in particle size and improvement in zeta potential was responsible for the

improvement in the solubility, which had probably reduced the time for dissolution kinetics. Increased pH showed significant ($P<0.05$) decrease in solubility of M34 samples (Table 3.5.3.3). Even though there was not a significant difference in the solubility of M70 and M80 samples during the storage time, means of M70 and M80 control samples showed a decrease in solubility (Table 3.5.3.4, and 3.5.3.5). However, we found a significant decrease ($P<0.05$) in the solubility of control and 7 pH treated M34 samples at both temperatures during the storage time (Table 3.5.3.6).

Table 3.5.3.3. Solubility (%) (mean ± SEM) of different treatments of M34, M70, and M80 samples. Values with the same superscript within a column are not significantly different ($P > 0.05$).

Treatment	pH	Heating (°C)	Solubility (%)		
			M34	M70	M80
Control	-	-	99.881^{ab}	99.614^{a}	99.204^{a}
N85	7	85	99.897^{ab}	99.661^{a}	97.467^{a}
H85	8	85	99.877^{ab}	99.790^{a}	99.57^{a}
HH85	9	85	97.756^{b}	99.892^{a}	99.838^{a}
N105	7	105	99.838^{ab}	97.107^{a}	95.855^{a}
H105	8	105	99.659^{ab}	99.307^{a}	98.892^{a}
HH105	9	105	-[1]	99.876^{a}	99.848^{a}
Pooled SEM			0.07	0.819	0.684

[1]No sample, treated milk coagulated before spray drying.

Table 3.5.3.4. Solubility (%) (mean ± SEM) of different treatments of M70 during storage period.

Treatments	Solubility (%)	
	0 day	20th day
M70	99.614	97.856
M70HH85	99.892	99.819
M70HH105	99.876	99.795
Pooled SEM	0.476	0.476

Table 3.5.3.5. Solubility (%) (mean ± SEM) of different treatments of M80 during storage period.

Treatments	Solubility (%)	
	0 day	20th day
M80	99.204	95.893
M80HH85	99.838	99.797
M80HH105	99.848	99.761
Pooled SEM	1.085	1.085

Table 3.5.3.6. Solubility (%) (mean ± SEM) of different treatments of M34 during storage period.

Treatments	Solubility (%)	
	0 day	20th day
M34	99.881	99.823
M34N85	99.897	99.844
M34N105	99.838	99.815
Pooled SEM	0.029	0.029

The gelling point (G' > G") was only noted in the dispersions of M70N85 (Figure 3.8.2) and M80N85 (Figure 3.8.3) samples at 41.7 and 53.8 minutes, respectively. As we observed an increase in zeta potential with an increased pH at both temperature in M70 and M80 samples, which probably deterred the aggregation of the CN in 8 and 9 pH samples, and thus prevented the rennet gelation during test time (Ferrer et al., 2008). Gelation of M70H105 and M80H105 samples did not occur probably due to the high heat denaturation of proteins (Ferrer et al., 2008). Control samples of M70 and M80 did not show any gelation. We had not observed the gelation in M34 sample treatments except the control sample (M34), which was 24.2 minutes (Figure 3.8.1).

3.6. Conclusions and future scope

The uplift of zeta potential in the alkaline pH treated samples was responsible for the weakening of CN micelle structure in M70 and M80 samples. Both high-pressure DSI and pH change disrupted the CN micelle and reduced the size of CN micelle. High zeta potential and smaller particle size improved the solubility in M70 and M80 samples. Free released caseins due to dissociation of CN micelle at alkaline pH increased the viscosity of samples in

M70 and M80 samples. Gelation time was prolonged, probably due to an increase in zeta potential. Means of M70 and M80 samples at 9 pH at both temperatures (85 and 105 °C) showed higher solubility as compared to control and other treatment samples. Whereas the opposite effect was observed on M34 samples. High pH treated powders could not be used in cheese manufacturing because of prolonged gelation time. Heat treatments at 9 pH have more applications to MPC than NFDM. 85 °C temperature treatment at higher pH for both MPCs seemed just as effective as 105 °C temperature treatment. Further research is required to check the functionality of yogurt, and other fluid systems such as dairy beverages, incorporated with high pH and DSI processed MPCs. Effect of using other chemicals to change the pH of UF retentate before DSI treatment can also be determined. Even though Friedman et al. (1981) states that at Lysinoalanine (LAL) formation happens only above the 9 pH but determine the LAL formation in the condition of DSI temperature treatment (85 ± 2°C and 105 ± 2°C for 33 ± 1 s) at 7, 8, and 9 pH could be different. Therefore, another study can be performed to determine LAL formation in these DSI and pH conditions. Furthermore, storage study can be performed to check the effect of storage time on all functional properties of DSI and pH treated MPCs. Limitation of this study is that NFDM and MPCs were manufactured in duplicate due to the cost of production and analysis. So, another study can be performed in which either MPC70 or MPC80 can be manufactured in triplicate, to increase the degree of freedoms, using the DSI temperature treatment (85 ± 2°C and 105 ± 2°C for 33 ± 1 s) at 9 pH with a control sample, and limited analysis can be performed in that study to decrease the cost of analysis and production. Functionality of high pH and DSI treated MPCs, along with control samples, could be checked during different periods of accelerated shelf-life study. Transmission electron microscopy can be used to give more

precise information on the type of aggregates formed during heating. Another study can be performed to check the heating effects on the morphology of the powder that allows better solubility. Same study can be performed using neutral pH water for diafiltration. Heat and acid induction gelation studies can be performed using high pH and DSI treated MPC powders.

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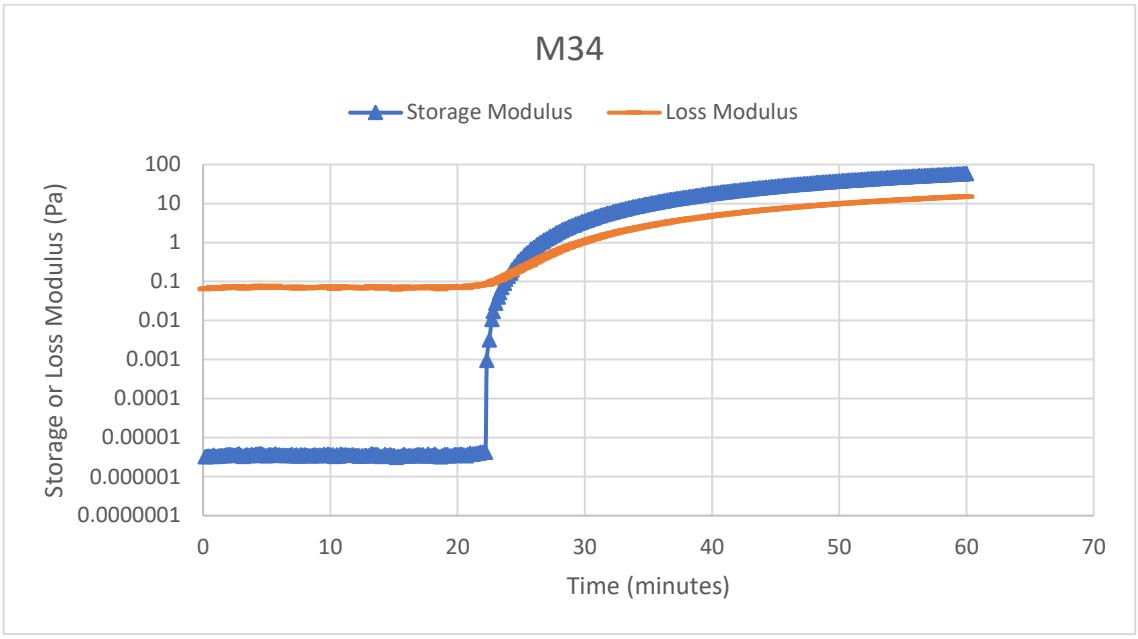
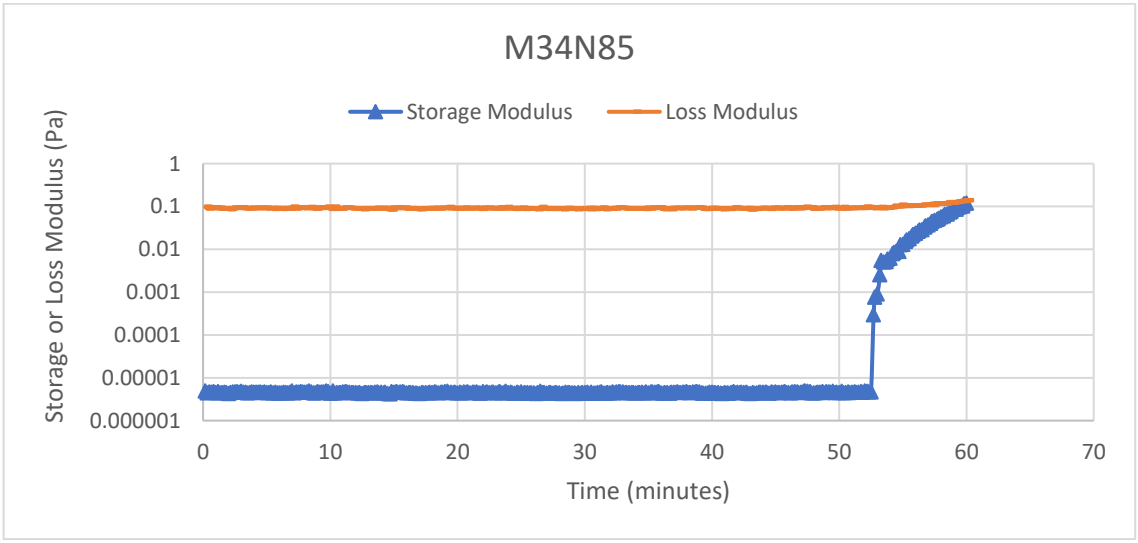
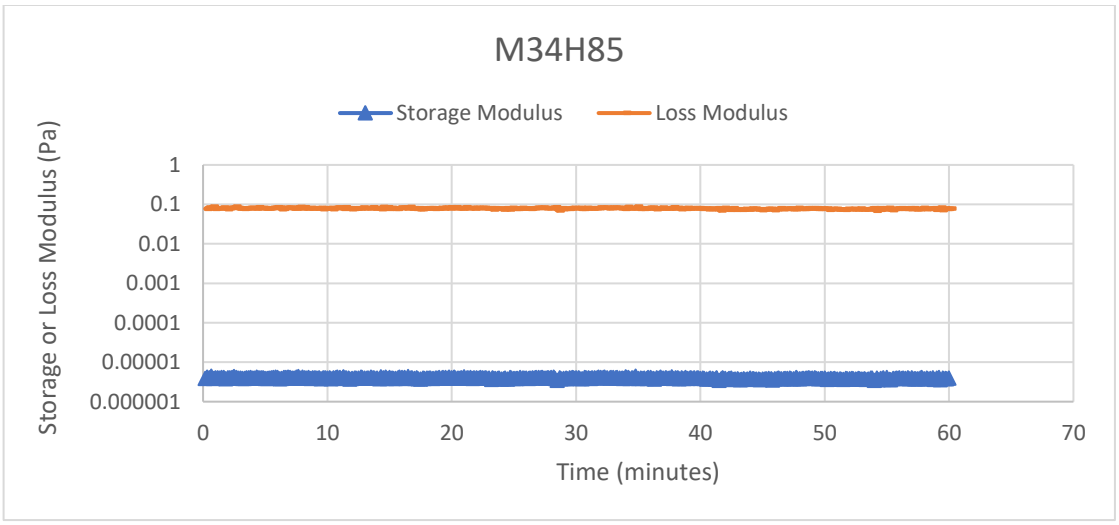
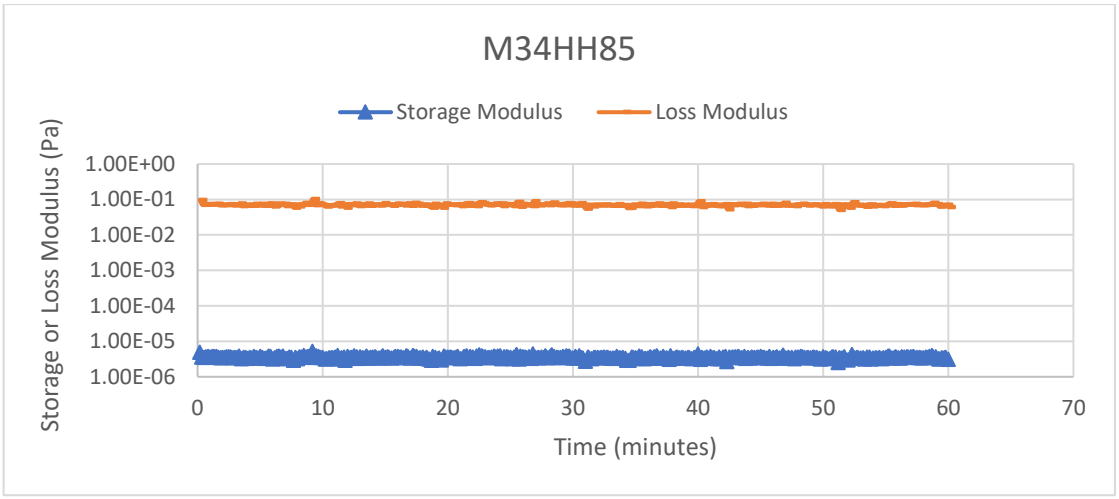
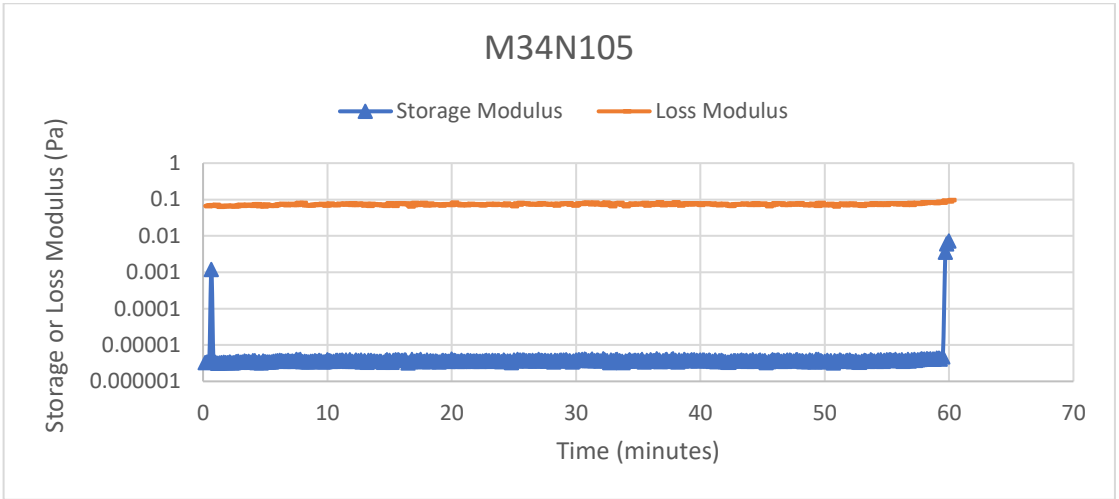
3.8. Appendix


M34
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
10
20
30
40
50
60
70
Time (minutes)


M34N85
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M34H85
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M34HH85
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
0
10
20
30
40
50
60
70
Time (minutes)


M34N105
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)

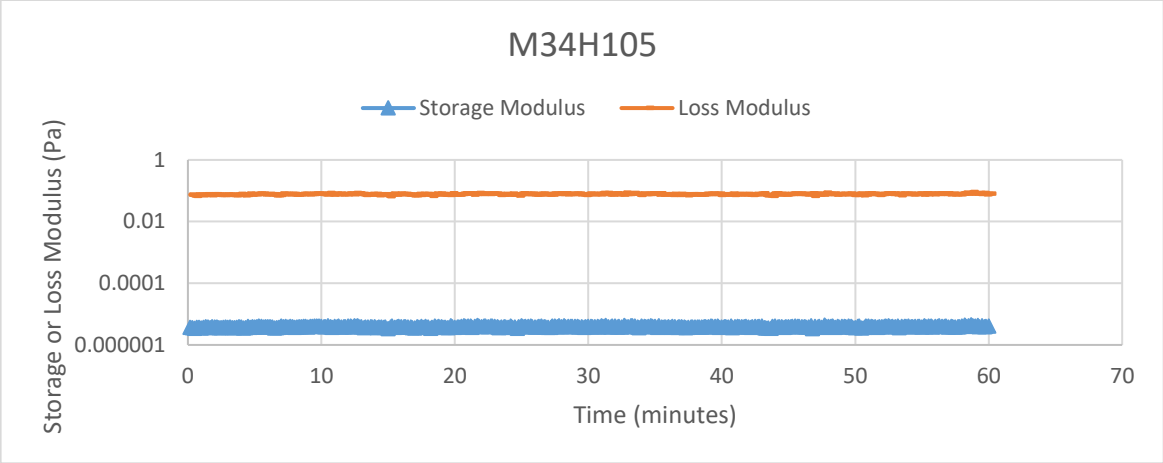
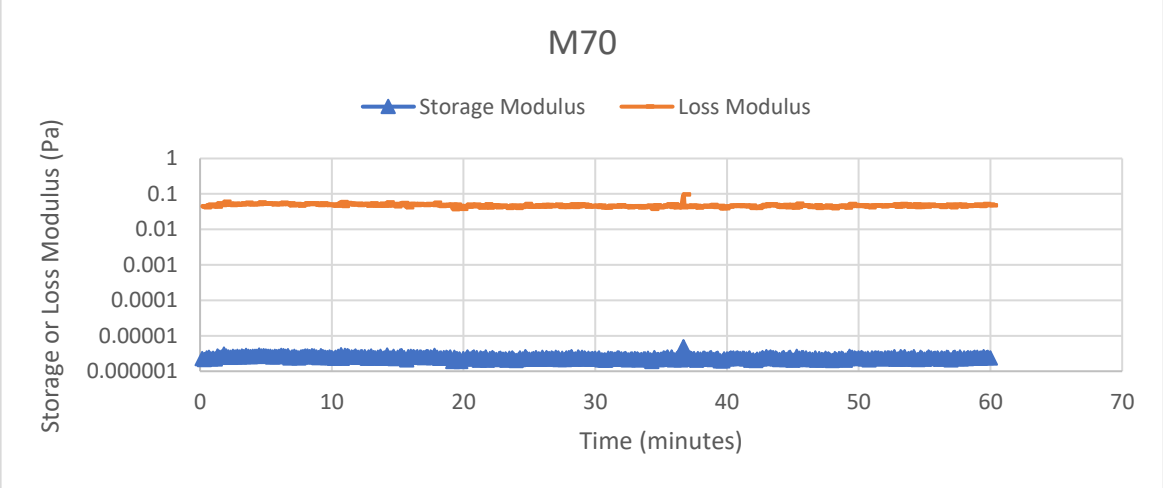
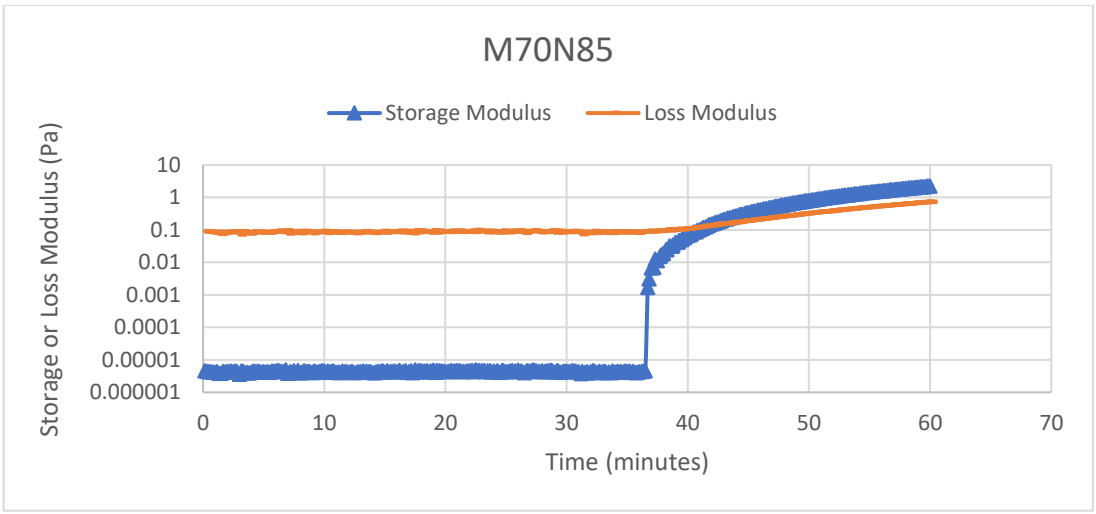
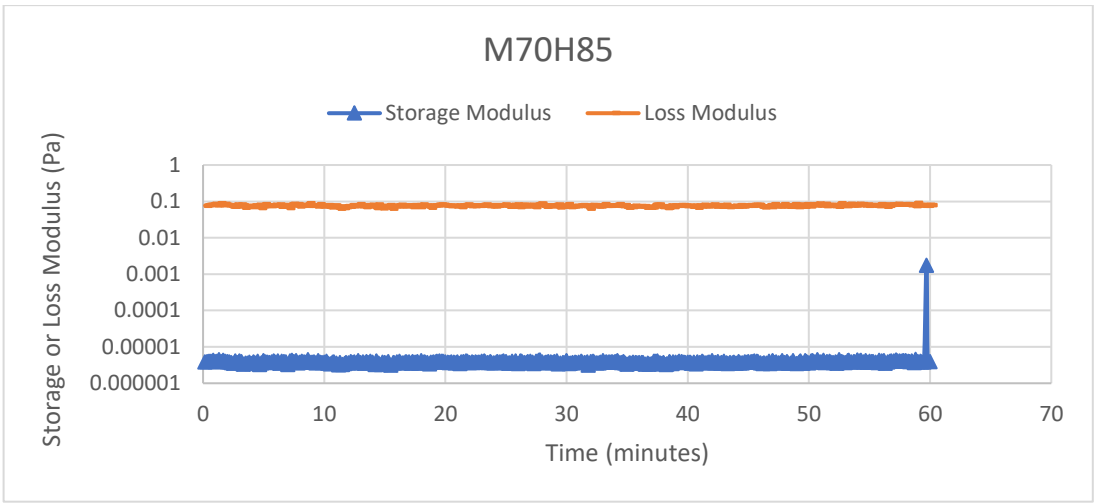
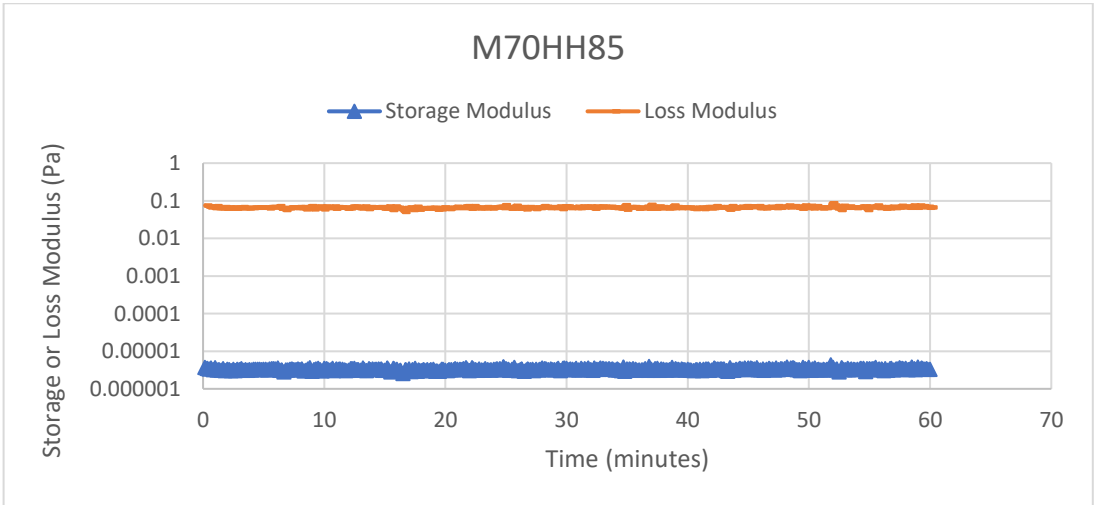


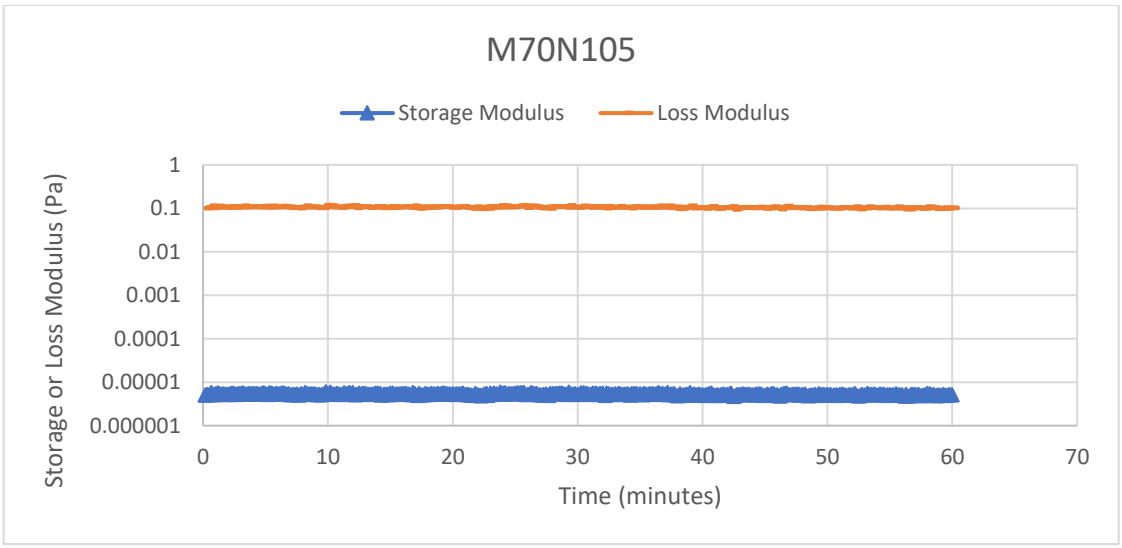
Figure 3.8.1. Gelation time (crossover point of storage and loss modulus) of different treatments of M34 samples using the amplitude sweep from 0.01 to 100% at 30ºC with a frequency of 1 rad/s.

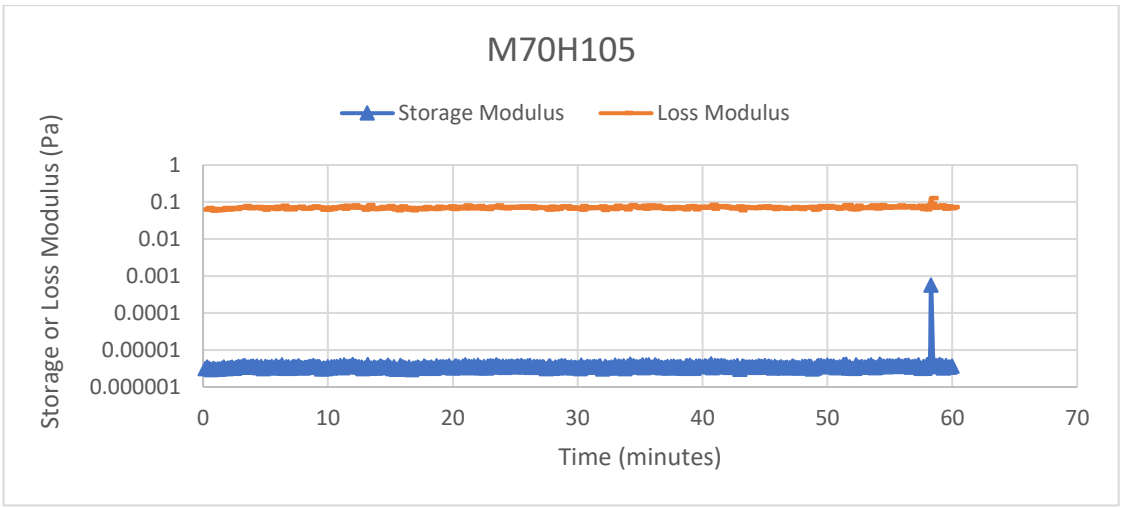



M70N85
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M70H85
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M70HH85
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M70N105
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M70H105
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)

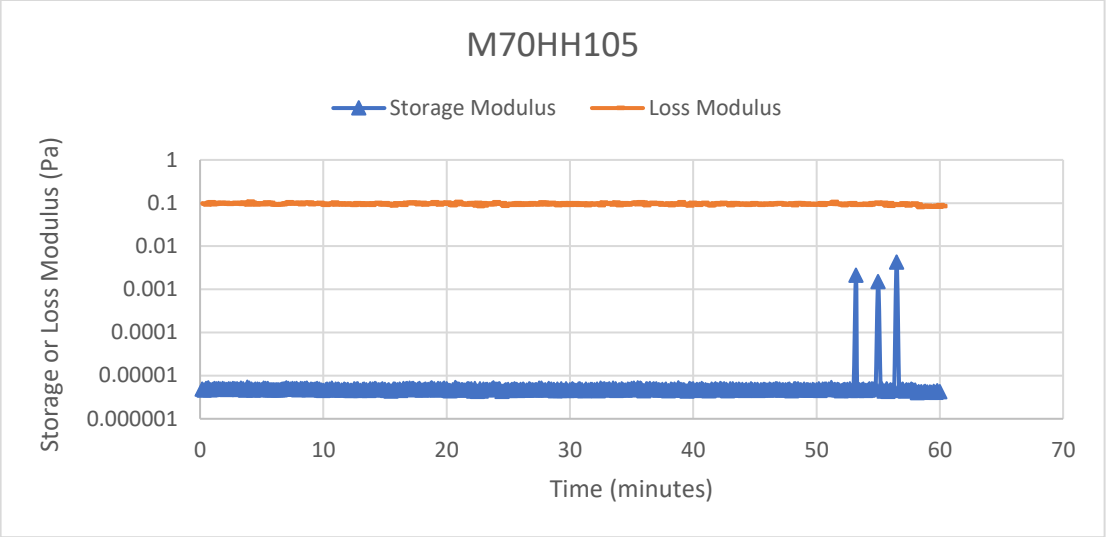
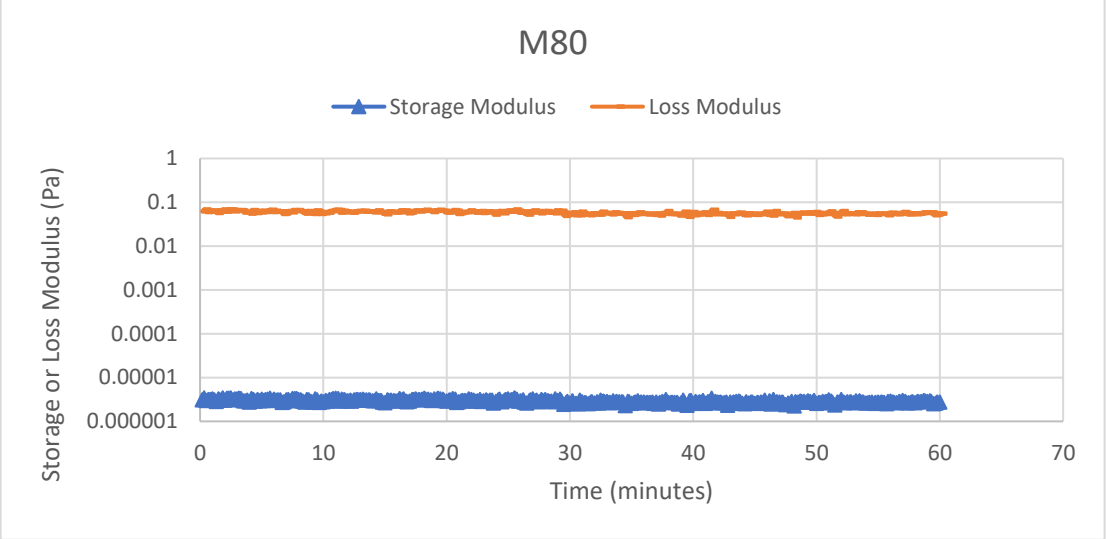
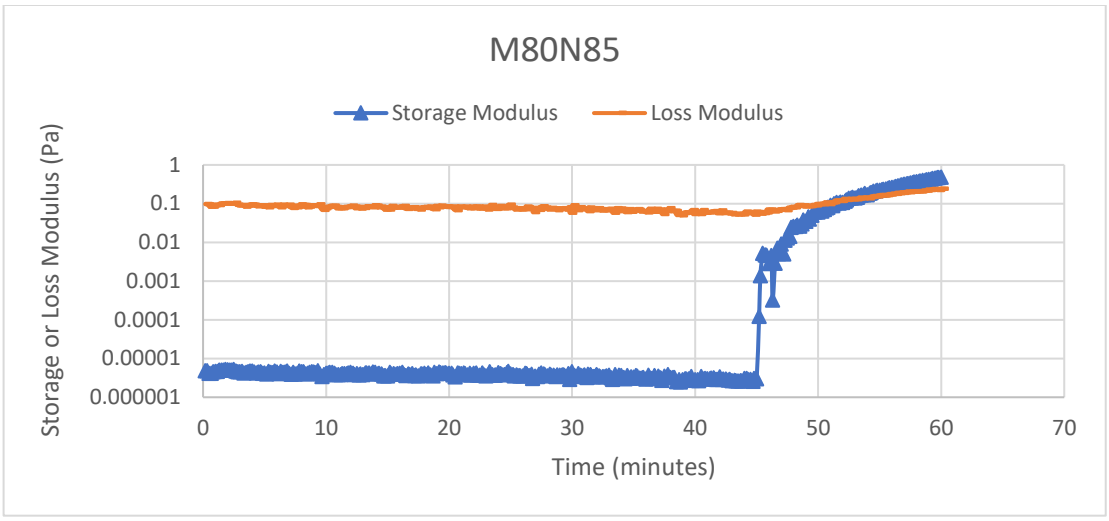
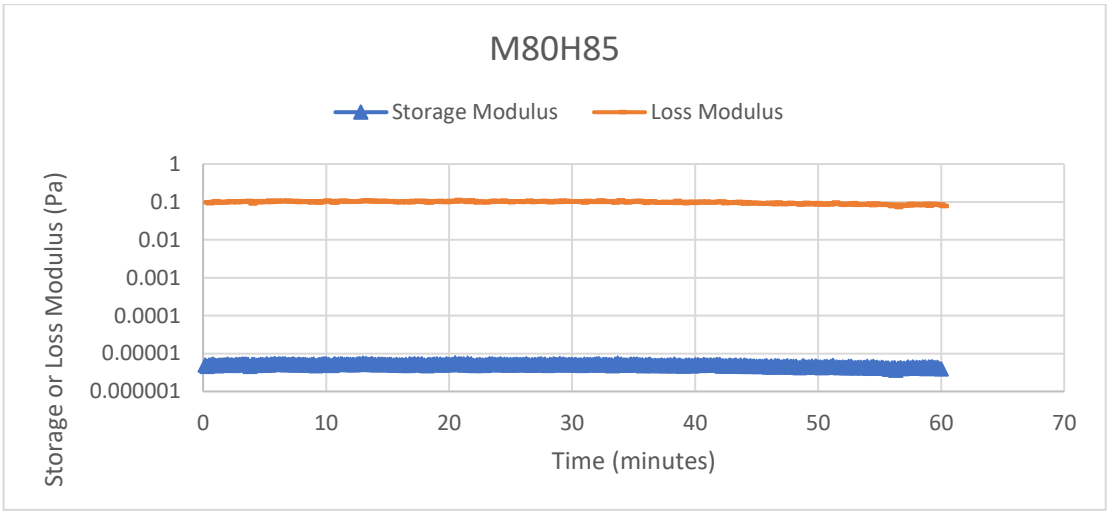
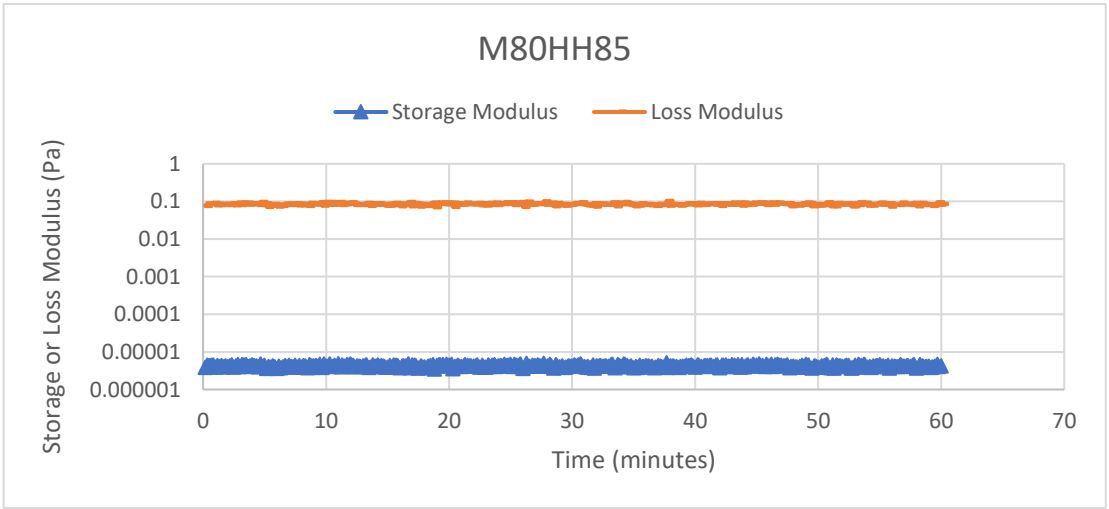


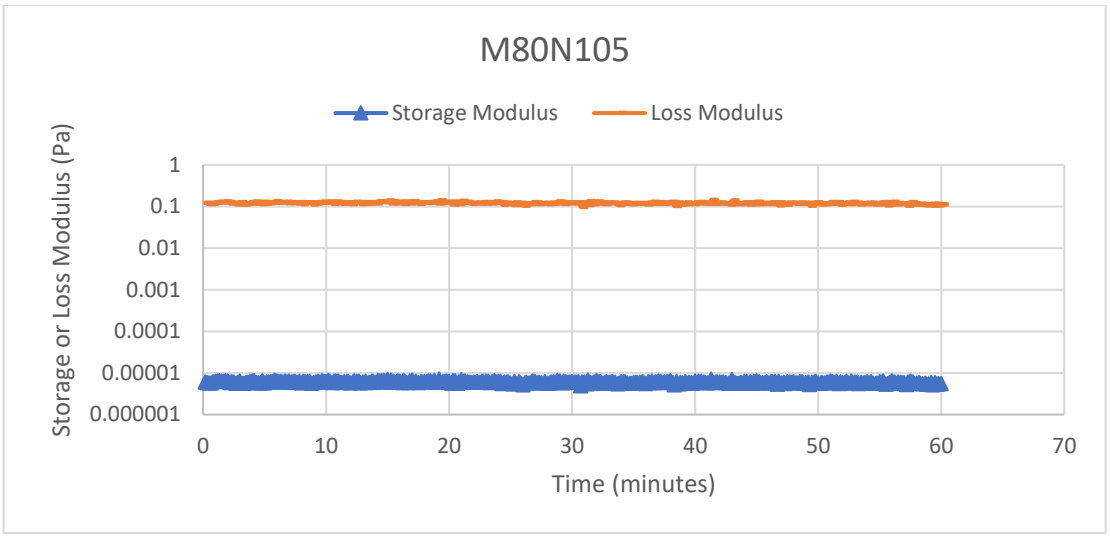
Figure 3.8.2. Gelation time (crossover point of storage and loss modulus) of different treatments of M70 samples using the amplitude sweep from 0.01 to 100% at 30ºC with a frequency of 1 rad/s.

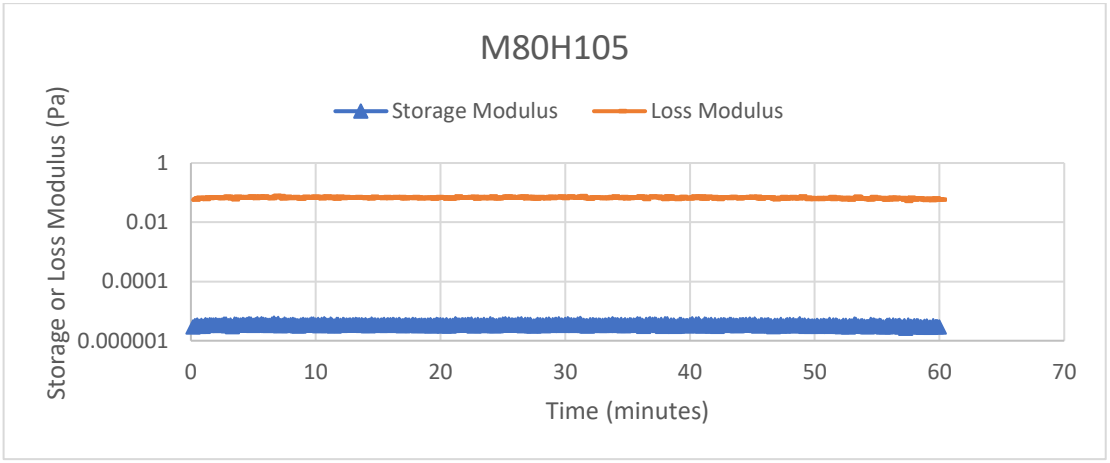



M80N85
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M80H85
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M80HH85
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M80N105
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)


M80H105
Storage Modulus
Loss Modulus
Storage or Loss Modulus (Pa)
1
0.01
0.0001
0.000001
0
10
20
30
40
50
60
70
Time (minutes)

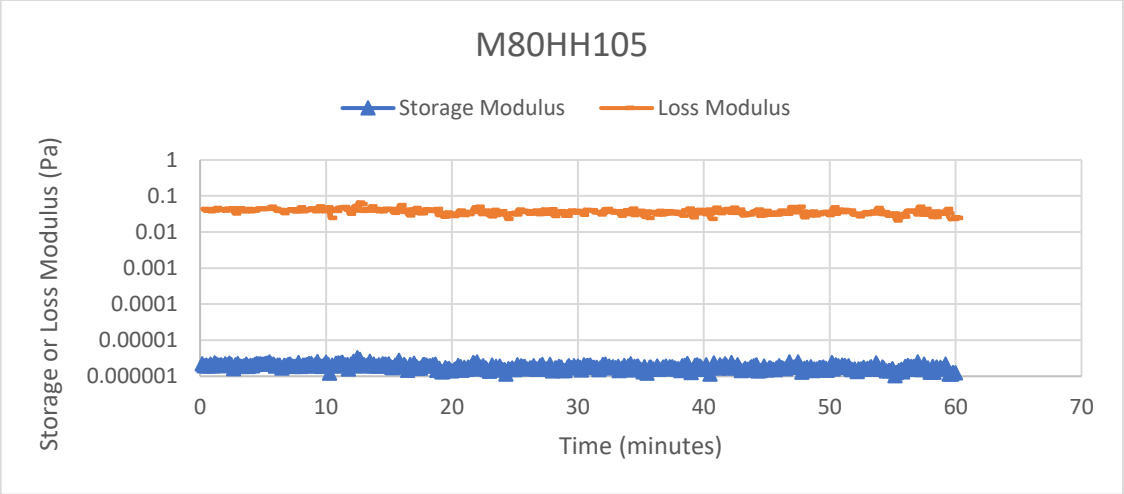


Figure 3.8.3. Gelation time (crossover point of storage and loss modulus) of different treatments of M80 samples using the amplitude sweep from 0.01 to 100% at 30ºC with a frequency of 1 rad/s.