Impact of Heating and Shearing on Physicochemical Properties of Reconstituted Milk Protein Concentrates

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(B.Sc., M.Sc.)

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Victoria University, Australia

Institute of Sustainability Industries and Liveable Cities

February 2024

Abstract

Reconstituted milk products, particularly milk protein concentrates (MPC), are becoming increasingly popular for their enhanced qualities and health benefits, playing a pivotal role in protein-rich dairy beverages. However, essential thermal treatments for ensuring safety, such as pasteurization and sterilization, along with shear forces from mechanical processing, can modify their physical, nutritional, and functional properties. Additionally, the stability of these dairy beverages is influenced by factors such as protein concentration, pH, and the addition of chelators and hydrocolloids during production. Therefore, the primary objective of this study was to investigate the impact of these factors on the structural and functional behaviour of milk proteins in MPCs, specifically under the combined effects of heat/time (90°C/5 min or 121°C/2.6 min) and shear rate (100, 1000, or 1500 s^{-1}).

MPC suspensions were subjected to heat and shear treatments using a rheometer equipped with a narrow-gap coaxial cylinder pressure cell. The secondary structural changes of the proteins were analysed using Fourier transform infrared (FTIR) spectroscopy. Protein denaturation and aggregation were assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC). Mineral analysis was performed using a calcium ion selective electrode and inductively coupled plasma optical emission spectrometry (ICP-OES). Particle size and zeta potential analyses were conducted using a Zetasizer.

The heat stability of reconstituted MPCs at different protein concentrations (4% and 8% w/w) was evaluated under combined heat and shear. The results demonstrated significant differences in heat stability between the two concentrations. For MPC suspensions with 4% protein, the impact of shear on heat stability was relatively minor. However, this effect became more pronounced in suspensions with an 8% protein concentration, leading to noticeable reductions in heat stability. The higher protein concentration in these suspensions facilitated increased protein-protein interactions under shear, resulting in greater protein aggregation at elevated temperatures. Consequently, this adversely affected the heat stability of these reconstituted MPC's.

Subsequent investigations were conducted utilizing an 8% MPC concentration, given the marked influence of shear on heat stability observed at this level. The second study explored the behaviour of milk proteins in suspensions adjusted to pH levels of 6.1, 6.4, 6.8, or 7.5, under combined heat and shear treatments. Under alkaline conditions, shear forces notably enhanced heat-induced micellar dissociation. Conversely, at pH 6.1, shear forces facilitated heat-induced aggregation, whereas at pH 6.4, the effect of shear predominantly led to the fragmentation of aggregates.

Further studies were focused upon common additives used in the dairy industry and their impact on the shear effect. The impact of calcium sequestering salts (CSS), disodium hydrogen phosphate (DSHP) and trisodium citrate (TSC), on the heat stability of milk proteins was analysed. Combined shear and heat were observed to significantly affect the milk system when CSS is present. Moreover, the lower calcium binding affinity of DSHP reduces micellar disruption, thereby enhancing the effect of shear.

Finally, the heat stability of milk protein dispersions with added cocoa powder (1.5% (w/w)), sucrose (7% (w/w)), and varying levels of κ-carrageenan (0.01, 0.03, or 0.05% (w/w)) under combined heat and shear conditions was examined. Shear forces led to considerable protein aggregation, particularly at higher κ-carrageenan concentrations. The aggregation was mainly due to micelle destabilization and the presence of loosely bound caseins within the κ-carrageenan network, rendering them more prone to aggregation as shear increased collision frequencies.

Shear forces present in dairy processing are critical in defining the attributes of the final product, thereby underscoring the necessity to meticulously consider these dynamics within the realm of dairy science research. This study significantly advances food science by examining how combined heat and shear treatments affect the properties of MPCs, essential for optimizing protein-rich dairy beverages. Understanding these dynamics leads to enhanced safety, quality, and functionality in dairy products, contributing to broader innovation and sustainability in the food industry.

Declaration

"I, Mediwaththe Gedara Anushka Thejangani Menike Mediwaththe, declare that the PhD thesis entitled **"Impact of Heating and Shearing on Physicochemical Properties of Reconstituted Milk Protein Concentrates"** is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

"I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures".

Signature:  Date: 21/02/2024

Acknowledgements

I extend my sincerest gratitude to my esteemed principal supervisor, Professor Todor Vasiljevic, whose exceptional guidance, unwavering support, and constant encouragement were fundamental to my journey throughout this study. His deep expertise, steadfast patience, and inspiring insights not only endowed me with the confidence to independently pursue my research but also significantly influenced my academic and professional development. It was an honour to have been under the mentorship of such a distinguished scholar, and I am deeply thankful for the opportunities and understanding he extended during this critical phase of my life.

I express my heartfelt gratitude to my co-supervisor, Professor Thom Huppertz, whose expertise and guidance have been invaluable throughout my research journey. His dedication to academic excellence and commitment to nurturing my development as a researcher were evident in every piece of advice he provided. It has been an immense privilege to work under the guidance of such a respected and accomplished scholar. I am deeply appreciative of the opportunities for growth and learning he facilitated.

I also extend my profound gratitude to my co-supervisor, Associate Professor Jayani Chandrapala, whose invaluable guidance and support have been pivotal to my research journey. Her dedication and expertise have significantly contributed to the success of my research. I am deeply grateful for the exceptional guidance she has generously provided.

I gratefully acknowledge Dr. Muditha Dissanayake, Dr. Samantha Liyanaarachchi, and Associate Professor Senaka Ranadheera for their helpful advice, suggestions, and technical support on numerous occasions.

Special thanks are also extended to Dr. Marlene Cran for editing and proofreading my thesis and for providing technical assistance with FTIR throughout my studies.

My gratitude extends to the technical staff at the Werribee campus for their exceptional support. I would like to acknowledge Stacey Lloyd, Melinda Tagiwasa, Chairmane

DiQuattro, Mary Marshall, Nishantha Illangantilaka, and Larruceo Bautista for their invaluable assistance.

I extend my deepest appreciation to my colleagues Rangani Wijayasinghe, Surjit Kaur, Elaheh Ahmadi, Tatijana Markoska, Joseph Kayihura, Geetha Vermula, Derrick D'Souza, Davor Daniloski, and all others for their support and friendship.

I am deeply thankful to my parents for their steadfast support and moral fortitude, which have been the foundation of my strength throughout my academic pursuits. Their unwavering faith in me has continuously inspired me.

I also extend my heartfelt gratitude to my husband, Sanvidha, for his remarkable devotion, patience, and encouragement, which have been crucial to my studies. His support has been pivotal throughout this demanding journey.

Finally, a heartfelt thank you to my sons, Savishka, Sanishka, and Sathishka, for their significant sacrifices at such tender ages. Their understanding and patience, despite the absence of their mother due to my academic obligations, have profoundly touched me.

DETAILS OF INCLUDED PAPERS: THESIS WITH PUBLICATION

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Chapter No.	Publication Title	Publication Status • Published • Accepted for publication • In revised and resubmit stage • Under review • Manuscript ready for submission	Publication Details • Citation, if published • Title, Journal, Date of acceptance letter and Corresponding editor's email address • Title, Journal, Date of submission
3	Effect of Protein Content on Heat Stability of Reconstituted Milk Protein Concentrate under Controlled Shearing	Published	Mediwaththe, A., Huppertz, T., Chandrapala, J., & Vasiljevic, T. (2024). Effect of Protein Content on Heat Stability of Reconstituted Milk Protein Concentrate under Controlled Shearing. Foods, 13(2), 263
4	Effect of pH and Shear on Heat-Induced Changes in Milk Protein Concentrate Suspensions	Published	Mediwaththe, A., Huppertz, T., Chandrapala, J., & Vasiljevic, T. (2024). Effect of pH and Shear on Heat-Induced Changes in Milk Protein Concentrate Suspensions. Foods, 13(10), 1517.
5	Heat-induced changes of milk protein concentrate suspensions as affected by addition of calcium sequestering salts and shearing	Published	Mediwaththe, A., Chandrapala, J., Huppertz, T., & Vasiljevic, T. (2024). Heat-induced changes of milk protein concentrate suspensions as affected by addition of calcium sequestering salts and shearing. International Dairy Journal, 149, 105829
6	Heat-Induced Changes in κ-Carrageenan-Containing Chocolate-Flavoured Milk Protein Concentrate Suspensions under Controlled Shearing	Published	Mediwaththe, A., Huppertz, T., Chandrapala, J., & Vasiljevic, T. (2023). Heat-Induced Changes in κ-Carrageenan-Containing Chocolate-Flavoured Milk Protein Concentrate Suspensions under Controlled Shearing. Foods, 12(24), 4494

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Signature:

Date: 16-05-2024

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List of Abbreviations and Units

BSA	bovine serum albumin
°C	degree Celsius
CCP	colloidal calcium phosphate
CN	casein
CN:WP	casein to whey protein ratio
CSS	calcium sequestering salts
Cys	cysteine
DSHP	disodium hydrogen phosphate
FTIR	Fourier transform infrared spectroscopy
GLM	general linear model
g	gram
h	hour
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
MCC	micellar casein concentrate
MCP	micellar calcium phosphate
MPC	milk protein concentrate
min	minute
mL	millilitre
mM	millimolar
MPa	mega pascal
NaOH	sodium hydroxide
PAGE	poly acrylamide gel electrophoresis
pH	hydrogen ion concentration
PSD	particle size distribution

RTD	ready to drink beverages
s	second
SAS	statistical analysis software
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SH	sulphydryl
SS	disulfide
SPI	serum protein isolate (milk)
TS	total solids
TSC	trisodium citrate
UHT	ultra-high temperature
v/v	volume per volume
w/w	weight per weight
WP	whey protein
WPC	whey protein concentrate
WPI	whey protein isolate
α-LA	α-lactalbumin
αs1-CN	αs1-casein
αs2-CN	αs2-casein
β-CN	β-casein
β-LG	β-lactoglobulin
μL	microlitre

CHAPTER 1. Introduction

1.1 Background

The utilization of dairy powders for the production of reconstituted milk products has significantly increased due to their versatility and strong consumer demand. MPCs commonly utilized in protein-enriched dairy beverages, are known for their favourable sensory characteristics, high protein content, and health-promoting properties. These properties make them suitable for various applications, including meal replacements for clinical nutrition, therapeutic interventions for conditions like sarcopenia, and the formulation of lactose-free milk alternatives. In these applications, MPCs are typically mixed with emulsifiers and/or flavours before being thermally processed by pasteurisation, retort sterilisation or ultra-high temperature (UHT) treatment to make them safe and shelf-stable (Liang, Patel, Matia-Merino, Ye, & Golding, 2013).

MPC is derived from skim milk through membrane filtration, a process that concentrates two primary protein groups (casein and whey proteins) while reducing non-protein components (Gesan-Guiziou, 2013). MPCs are categorized based on their protein content, with typical levels ranging from ≥40% to ≤89% (Meena, Singh, Panjagari, & Arora, 2017). The membrane filtration method largely preserves the native state of these proteins with minimal formation of covalently bonded aggregates, thus maintaining the natural ratio of casein to whey proteins (78:22) (Crowley, 2016). However, changes in the concentration of other constituents, such as minerals, non-protein nitrogen, and lactose, relative to protein, have been indicated to impact the heat stability of reconstituted milk due to the concentration factor achieved during membrane filtration (Huppertz, 2016).

Despite significant research into the heat stability of reconstituted MPCs, limited understanding exists regarding the effects of other common processing parameters, particularly shear forces in conjunction with temperature. Shear forces are involved in several processing steps, including pumping, stirring, mixing, homogenizing, and passing through heat exchangers during heating, holding, and cooling. These forces have a direct impact on protein stability and the mineral environment within the milk system (Mediwaththe, Bogahawaththa, Grewal, Chandrapala, & Vasiljevic, 2018; Mediwaththe, Chandrapala, & Vasiljevic, 2018). The shear forces induce significant velocity gradients during fluid flow, causing conformational changes or even unfolding and exposure of

hydrophobic amino acids. This, in turn, induces protein interactions that ultimately lead to aggregation (Di Stasio & De Cristofaro, 2010). Increased flow rates under hydrodynamic shear conditions can lead to orthokinetic aggregation instead of the typical perikinetic aggregation driven by Brownian motion. This alternative aggregation process can result in various adverse outcomes, including changes in sensory perception due to modified food texture, phase inversions, and the formation of gels due to changes in size and morphology of protein structures (Walkenström & Hermansson, 1998). The aforementioned examples are among the very few studies reporting the protein stability of dairy fluids under combined heat and shear with variable physico-chemical environments such as changes to protein content, pH, addition of flavours or chemical substances such as chelators. This underscores the importance of studying proteins under such conditions, particularly focusing on protein concentrations commonly employed in commercial beverage production. Analysing the effects of these conditions on the structural changes and behaviour of predominantly intact native proteins would enhance our understanding of how these factors influence the physicochemical properties of MPCs and, consequently, the overall quality of the product. Moreover, such research could facilitate process enhancements or the development of novel processing methods by either reducing adverse changes in proteins or by enhancing process control to prevent or induce molecular interactions. Therefore, this thesis aims to address these knowledge gaps by investigating the effects of shear in conjunction with temperature on the heat stability of reconstituted MPCs under various parameters including protein content, pH, addition of calcium chelators and hydrocolloids. The novelty of this research lies in its comprehensive analysis of how these processing parameters influence protein stability and the overall quality of dairy products, providing valuable insights for dairy industry. In this study, several analytical methods were used to analyse protein structural changes based on their established efficacy. Secondary structural changes of proteins were analysed using FTIR spectroscopy, while SDS-PAGE and HPLC were employed to evaluate protein denaturation and aggregation. These methods collectively provide a comprehensive understanding of how processing parameters affect the structural integrity of proteins in reconstituted MPCs.

1.2 Research aims and objectives

The overall aim of the current study is to investigate the influence of heat and shear on the physicochemical properties of milk protein concentrates.

The specific objectives are:

- To establish a knowledge base in relation to the impact of combined heat and shear on the modification of native protein fractions of reconstituted MPC and their interactions.
- To relate the modification of native protein fractions induced by combined heat and shear to an altered physico-chemical environment within the milk system.
- To assess how these alterations within the system influence the protein-mineral environment and functionality of reconstituted MPC.
- To ascertain optimum process conditions which would result in minimal heat instability and enhance the functionality of reconstituted MPC under different physico-chemical environments.

1.3 Thesis Outline

This thesis has been organised into seven chapters. Chapter 1 discusses the aims of the study with the statement of significance including its contribution to the existing knowledge and practice. Chapter 2 reviews the literature relevant to the thesis and provides an overview of process induced modifications of reconstituted MPCs under controlled heating and shearing within different physico-chemical environments. Chapter 3 examines the impact of heat and shear on the heat stability of reconstituted 4% and 8% MPCs. Chapter 4 discusses the influence of pH on the heat stability of MPCs under controlled shearing. Chapter 5 details the effects of the addition of calcium chelators on the heat stability of MPCs under controlled heating and shearing. Chapter 6 examines the impact of shear on heat stability of chocolate flavoured milk protein concentrates under controlled heating and shearing. Chapter 7 concludes the research findings, reiterates the consequences and significance of the study, and details the scope for future work.

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CHAPTER 2. Literature Review

2.1 Bovine Milk

Milk, a fundamental component of the human diet worldwide, is an intricate biofluid composed of water (~87%), lactose (~4-5%), proteins (~3%), fats (~3-4%), minerals (~0.8%), enzymes (e.g., peroxidase, catalase, phosphatase, lipase) and a range of vitamins (~0.1%) such as vitamins A, C, D, thiamine and riboflavin (Pereira, 2014; Walstra, Wouters, & Geurts, 2006). The protein fraction of bovine milk, a crucial element of global nutrition, is mainly categorized based on solubility at a pH of 4.6. This categorization divides proteins into insoluble caseins, which constitute ~80% of total milk proteins, and soluble whey proteins, accounting for the remaining ~20% (Huppertz, Fox, de Kruif, & Kelly, 2006; Pereira, 2014). Each protein type exhibits unique physico-chemical characteristics.

2.2 Caseins

Caseins (CN), distinguished by their unique structural and functional attributes, are classified into four main types: α_{S1}-, α_{S2}-, β-, and κ-CN (Bordin, Raposo, De la Calle, & Rodriguez, 2001; Creamer & MacGibbon, 1996). Each of these casein types exhibits distinct primary structures and are characterized by several genetic variants (Creamer & MacGibbon, 1996). Around 95% of caseins exist in a micellar form, of which 94% are proteins, and the remaining dry weight is colloidal calcium phosphate (Fox, 2003).

Caseins exhibit flexible and open conformations (Perticaroli, Nickels, Ehlers, Mamontov, & Sokolov, 2014; Smyth, Clegg, & Holt, 2004). Unlike whey proteins, caseins demonstrate elevated surface hydrophobicity. Their primary structure, characterized by a high proline content, results in minimal α-helix or β-sheet structures (Creamer & MacGibbon, 1996). Notably, the distribution of polar and apolar residues in caseins is non-uniform, forming clusters that create distinct hydrophobic and hydrophilic regions (Creamer & MacGibbon, 1996; Slattery & Evard, 1973). Among caseins, β-CN stands out as the most hydrophobic, while α_{S2}-CN is identified as the most hydrophilic. The C-terminal region of κ-CN exhibits strong hydrophilicity, contrasting with the hydrophobic N-terminus, which is a crucial factor contributing to micelle stability (Creamer & MacGibbon, 1996; Slattery & Evard, 1973).

The κ-CN and α_{S2}-CN types can form intermolecular disulfide linkages with whey proteins, (Azuma et al., 1994; Rasmussen et al., 1999). In its native state, κ-CN can range in size from a monomer to multimeric disulfide-linked structures larger than a decamer (Bouguyon, Beauvallet, Huet, & Chanat, 2006). The multimeric nature of κ-CN is thought to facilitate its coverage of the micelle surface, thereby contributing to the stabilization of the micelle structure (Rasmussen, Højrup, & Petersen, 1992). Conversely, β-CN and α_{S1}-CN, do not contain cysteine (Jang & Swaisgood, 1990) and are therefore unable to form disulfide bonds. A summary of the characteristic properties of the four types of milk caseins is shown in Table 2.1.

Table 2.1 Characteristics of four types of caseins in milk*

Characteristic	Type of casein			
	α_{S1}-CN	**α_{S2}-CN**	**β-CN**	**κ-CN**
Content in milk (%)	1.2-1.5	0.3-0.5	0.9-1.1	0.3-0.4
No. of amino acid residues	199	207	209	169
No. of cysteine residues	0	2	0	2
No. of proline residues	17	10	34-35	20
No. of phosphate groups	2	3	1	0
Molecular weight (Da)	~23000	~25000	~24000	~19000

*Adapted from Creamer & MacGibbon (1996), De Kruif & Holt (2003b)

2.2.1 Casein micelles

Nearly 95% of caseins in normal milk are present as micelles which are spherical colloidal particles, demonstrating a broad range of variations in composition, structure, size distribution, and salt composition (Azuma et al., 1994; Smyth et al., 2004). Ranging in size from 50-300 nm, micelles possess a hydrated density within 1.06-1.11 g/cm and a voluminosity of 4.0-4.4 mL/g of casein, containing around 3.3-3.7 g of water per 1 g of casein (Azuma et al., 1994; C Holt, Carver, Ecroyd, & Thorn, 2013).

The dynamic nature of casein micelles is underscored by their composition, consisting of 94% protein and 6% small ions. Predominantly calcium, phosphate, magnesium, and citrate, these ions play a crucial role in maintaining micellar integrity under certain study conditions (Horne, 2006; Rasmussen et al., 1999). The composition of casein micelles

varies with temperature, pH, ionic strength, and water activity (Ruettimann & Ladisch, 1987; Walstra, 1999).

The stability of casein micelles in liquid milk colloidal suspensions is attributed primarily to κ-CN. Located on the external surface of the micelle, κ-CN extends hair-like strands that prevent micelle precipitation. The thermal stability of casein micelle structures is noteworthy, as they can withstand freezing, heating, and drying without compromising colloidal stability upon reconstitution to normal conditions (De Kruif & Holt, 2003b; Walstra, 2003). The distribution of caseins within casein micelles varies, with almost 90% present at 32°C, while at 4°C, up to 50% may be in the serum phase, illustrating the dynamic nature of casein micelles (De Kruif & Holt, 2003a).

2.2.2 Structure of casein micelles

The intricate internal arrangement of the native casein micelle remains a subject of debate (Dickinson, 2006). It is generally acknowledged that κ-CN plays a crucial role by forming a layer on the outer surface of the assembly, ensuring stability through a steric stabilization mechanism. In fact, κ-CN has the capability to stabilize approximately ten times its own mass of calcium-sensitive caseins (Fox & Kelly, 2012).

The primary models describing the structure of the casein micelle include the submicelle model and the dual binding model. In the former model, initially proposed by Waugh & Talbot (1971), the casein micelle is comprised of small aggregates known as submicelles. Each submicelle contains 15 to 20 casein molecules and has a diameter of 10-15 nm. These submicelles are formed by the linkage of calcium phosphate and α_S- or β-CN's through phosphoserine residues. Acting as a binding agent, colloidal calcium phosphate (CCP) cements the numerous submicelles together to create the complete casein micelle structure. κ-CN is situated on the micelle surface, with its hydrophobic part bound to the core of the micelle. Simultaneously, the hydrophilic macropeptide forms a protruding hairy layer, at least 7 nm thick, extending into the aqueous phase. This hairy layer serves to limit micelle aggregation by enhancing steric repulsion between micelles. While the submicelle model explains key aspects of the casein micelle, it faces limitations, particularly in clarifying the segregation of κ-CN on the micelle surface (Horne, 2006). Moreover, recent electron microscopy studies have not confirmed the presence of submicelles (Dalgleish, 2014).

Holt (1998) proposed an alternative model characterized by a more open and fluid structure, resembling a “bowl of spaghetti” (see Figure 2.1). In this model, polypeptide chains in the core are partially cross-linked by nanometer-sized clusters of calcium phosphate, with the interaction sites on the caseins being the phosphoseryl clusters of the calcium-sensitive caseins (Holt, 1998; Horne, 2006). According to this model, the casein micelle takes on the form of a tangled web of flexible casein molecules, creating a gel-like structure. However, a notable limitation of this model is the absence of a mechanism to restrict gel growth, and it does not assign a substantial role to κ-CN (Horne, 2006).

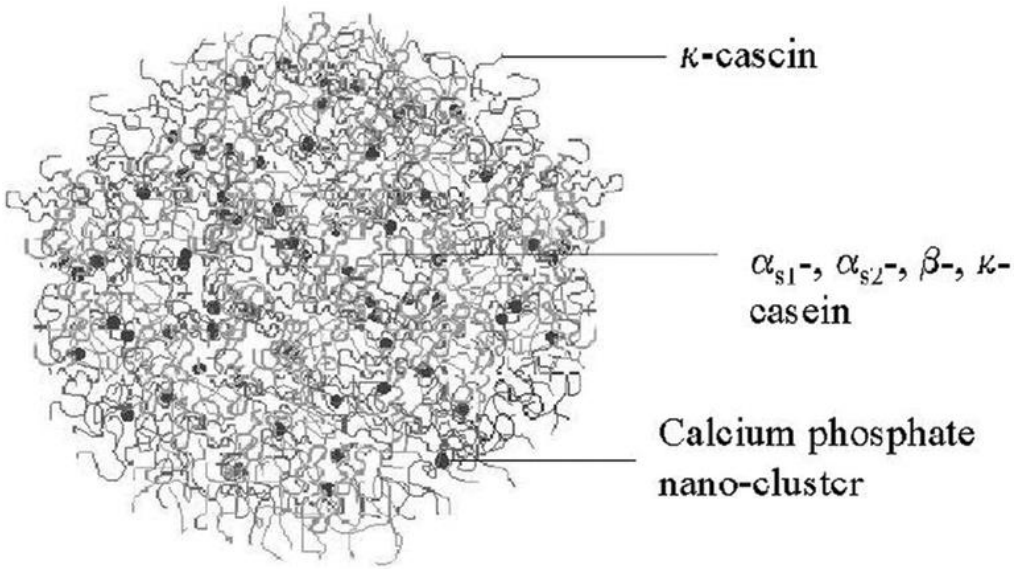

Figure 2.1 Schematic representation of Holt’s casein micelle structure from De Kruif & Holt (2003a, 2003b)

According to the dual binding model proposed by Horne (1998), micelle growth involves two distinct types of bonding: cross-linking through the hydrophobic regions of the caseins and bridging with calcium phosphate clusters. According to this model, α_{S1}-CN features two hydrophobic regions and one hydrophilic region, including the cluster. α_{S2}-CN possesses two hydrophobic regions and two clusters, whereas β-CN has only one hydrophobic group and one phosphoseryl group. The different caseins create a network by combining interactions between two hydrophobic regions and between two hydrophilic regions. The growth of the casein micelle is regulated by κ-CN, which can interact with other caseins through its hydrophobic terminal region but since it lacks a phosphoseryl cluster, this prevents secondary interactions. Consequently, κ-CN is located

on the micelle's surface and fulfills its role of providing a hairy stabilizing layer (Horne, 2006).

2.3 Whey proteins

Whey proteins are heterogeneous globular proteins characterized by clearly defined secondary and tertiary structures found in milk (Anema, 2020). The four primary whey proteins found in milk are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), and immunoglobulin (Ig) (Anema & Klostermeyer, 1996; Bordin et al., 2001). These proteins exhibit high hydrophobicity and have densely folded peptide chains that are sensitive to heat and undergo denaturation at temperatures exceeding 60°C (Corredig & Dalgleish, 1996; Creamer & MacGibbon, 1996) where the heat sensitivity order has been reported as Ig > BSA > β-LG > α-LA (Singh & Waungana, 2001). Table 2.2 provides a summary of the key attributes associated with major whey proteins.

Table 2.2 Characteristics of individual whey proteins*

Characteristic	α-LA	β-LG	BSA	Ig
Content in milk (%)	0.01-0.15	0.2-0.4	0.01-0.04	0.014-0.1
Native confirmation	Globular	Globular	Globular	Globular
No. of amino acid residues	123	162	607	-
No. of proline residues	8	8	28	-
No. of cysteine residues	8	5	35	-
Molecular weight (kDa)	14.1	18.4	69	150-161

*Adapted from Sawyer (2012)

2.3.1 β- lactoglobulin

β-LG is the most abundant whey protein constituting ~50% of the whey composition. It is a typical globular protein characterized by hydrophilic amino acids arranged on the exterior and hydrophobic amino acids positioned internally within its three-dimensional structure as depicted in Figure 2.2. Beta-LG is a polypeptide chain composed of 162 amino acid residues with two disulfide bonds (Cys66–Cys160 & Cys106–Cys119), a free thiol group (Cys121) and a molecular weight of around 18.3 kDa (Considine, Patel, Anema, Singh, & Creamer, 2007). The presence of β-LG in either monomeric or dimeric form depends on factors such as pH, ionic strength, and temperature (Wijayanti, Bansal,

& Deeth, 2014). Several studies using different methods have characterized the secondary structure of β-LG with circular dichroism spectroscopy 15% α-helix, 50% β-sheet, and 15-20% reverse turn structures (Creamer, Parry, & Malcolm, 1983). Fourier-transform infrared (FTIR) spectroscopy has provided additional insights, indicating 9-11% α-helix, 51-55% β-sheet, 20-27% β-turn, and 9-11% random coil structures (Dong et al., 1996). At temperatures between 60 and 65 °C, a significant alteration in the conformation of the molecule takes place which reveals the previously concealed -SH group of Cys121. This subsequently triggers sulfhydryl/disulfide (SH/S-S) interchange reactions that result in irreversible aggregation/polymerization (Considine et al., 2007).

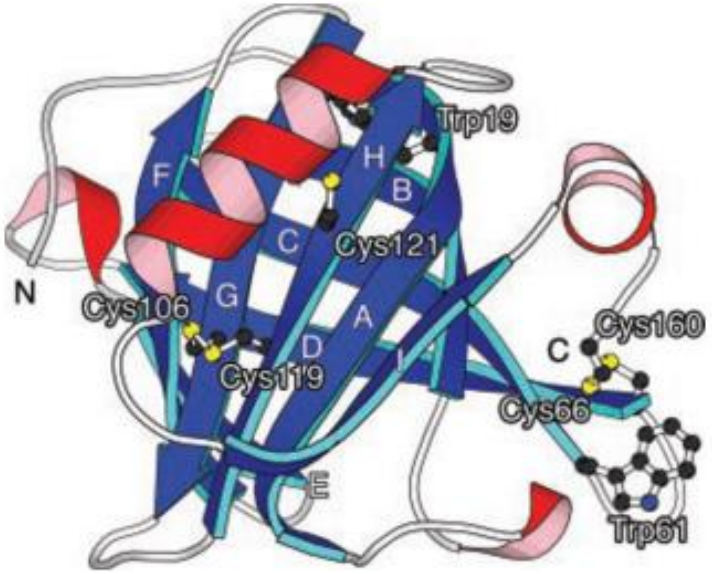

Figure 2.2 Crystal structure of bovine β-LG
from Yagi, Sakurai, Kalidas, Batt, & Goto (2003)

2.3.2 α-lactalbumin

α-LA is a smaller globular protein with a molecular weight of 14.2 kDa that is composed of 123 amino acid residues. Compared to other whey proteins, α-LA exhibits relatively high stability (Considine et al., 2007), with a primary structure including four disulfide bonds but lacking free thiol groups (Livney et al., 2003). Consequently, denatured α-LA can only integrate into aggregate structures via thiol-disulfide exchange reactions with denatured proteins containing free sulfhydryl groups (Oldfield, Taylor, & Singh, 2005). Although α-LA does not independently associate with casein micelles, it forms

associations with micelles through aggregates of β-LG. Therefore, the association behaviour of α-LA is likely influenced by that of β-LG (Corredig & Dalgleish, 1996).

2.4 Milk salts

Milk contains a diverse array of salts as summarised in Table 2.3. Among these salts, calcium and phosphate play crucial roles in preserving the structure of the casein micelle (Walstra et al., 2006). Salts with the ability to permeate through a membrane, with a molecular weight cutoff below 10,000-15,000 Da, are termed diffusible salts (Gaucheron, 2005), whereas salts that are retained by the membrane are recognized as being associated with the casein micelles.

Table 2.3 Salt composition in milk*

Type of salt	Total salt content (mM)	Diffusible salt content (mM)
Potassium	38.3	38.0
Chloride	30.4	30.3
Calcium	29.0	10.2
Sodium	22.0	22.0
Phosphate	20.6	12.4
Citric acid	9.5	9.4
Magnesium	4.9	3.4
Sulphate	1.2	1.2

*Adapted from Holt (2004); Little & Holt (2004)

2.5 Heat stability of milk

Thermal processing is commonly employed to improve the functional and sensory characteristics of milk in addition to microbiological safety (Kilic-Akyilmaz, Ozer, Bulat, & Topcu, 2022). The capacity of milk to endure elevated processing temperatures without excessive thickening and coagulation plays a pivotal role in the production of dairy products. The heat stability of milk constitutes a highly intricate phenomena, marked by numerous physical and chemical transformations in milk proteins during processes involving high temperatures. These transformations may manifest as an augmented viscosity or coagulation of the milk. Various compositional factors influence heat

stability, and the interplay among these factors complicates the assessment of individual significance, given that changes in one factor invariably impact others (O'connell & Fox, 2003). Alterations to heat stability can be achieved through the introduction of additives and adjustments to processing conditions (Fox, 1981). Although the precise mechanism underlying heat coagulation remains elusive, extensive investigations have been conducted to explore the effects of diverse compositional factors and treatment processes on heat stability as summarised in Table 2.4.

Table 2.4 Typical heat treatments applied in the dairy industry*

Heat treatment	Conditions
Thermization	65 ℃ for 15 min
Low-temperature long-time (LTLT) pasteurization	63 ℃ for 30 min
High-temperature short-time (HTST) pasteurization	72 ℃ for 15 s
Forewarming	90 ℃ for 2-10 min./ 120℃ for 20 s.
Ultra-high temperature (UHT) processing	130-140 ℃ for 3-5 s
In-container sterilization	110-115 ℃ for 10-20 min

*Adapted from O'connell & Fox (2003)

2.5.1 Denaturation of whey proteins

Whey proteins maintain their native conformation within relatively narrow pH and temperature ranges. However, subjecting them to extreme conditions of either temperature or pH leads to protein denaturation (Anema, 2000; Corredig & Dalgleish, 1996). The heat-induced modification of whey proteins can manifest as either reversible or irreversible transformations. Reversible modifications involve the partial unfolding of proteins, resulting in the loss of the helical structure. Irreversible changes trigger an aggregation process that includes thiol (–SH)/disulfide (S–S) interchange reactions and various intermolecular interactions such as hydrophobic and electrostatic interactions. In addition to interactions among whey proteins β-LG, α-LA, and BSA, there is potential interaction with cystine-containing caseins, particularly κ-CN. Moreover, evidence suggests that the thermal denaturation behaviour of individual whey proteins varies significantly (Anema & Li, 2003a).

Temperature governs both the rate and extent of denaturation in whey proteins, whereas pH regulates the interaction of whey proteins with casein micelles. Denaturation levels are known to escalate with rising temperatures (Anema & Li, 2003a). Prolonged heating at low temperatures or a gradual heating rate to a required temperature may facilitate the formation of denatured β-LG. Whey protein unfolding during heating exposes side-chain groups of amino acid residues that are normally concealed within the native structure, resulting in increased reactivity of certain groups (Jiménez-Guzmán et al., 2002; Oldfield, Singh, Taylor, & Pearce, 1998). Contrary to globular whey proteins, the open-structured casein proteins exhibit high heat stability. Nevertheless, cysteine residues of κ-CN and occasionally α_{S2}-CN may engage in sulfhydryl-disulfide interchange reactions with denatured whey proteins (Corredig & Dalgleish, 1996; Rasmussen et al., 1999). Non-covalent interactions, such as hydrophobic, ionic, and van der Waals forces, also play a role (Law & Leaver, 1997, 2000). Prior to the intermolecular disulfide formation, non-covalent complexes are formed (Jang & Swaisgood, 1990). It is important to consider that not all denatured whey proteins form complexes with the casein micelles; some remain in the serum, where they may aggregate with other whey proteins or with serum κ-CN (Anema, 2000; Oldfield et al., 2005).

In β-LG, the activation energy of denaturation decreases with increasing pH, whereas no clear pattern was observed for α-LA (Oldfield et al., 2000). Repulsion among ionized groups within the protein molecule has probably resulted in decline in activation energy for β-LG at higher pH. However, despite aiding in protein unfolding (denaturation), these charged groups impede intermolecular interactions (aggregation) due to their charge repulsion. β-LG carries a net negative charge at the pH of natural milk, so lowering the pH would reduce this charge, promoting aggregation (Law & Leaver, 2000; Oldfield et al., 2000). When the pH is increased, the activation energy of β-LG denaturation is decreased for disulfide-linked aggregation which may signify the importance of protein unfolding in generating thiol/disulfide groups capable of forming intermolecular disulfide bonds. Additionally, a conformational transition occurs at pH 6.9, leading to an increase in thiol group activity that could facilitate disulfide-linked aggregation.

The net charge of proteins plays a crucial role in determining the frequency of favourable collisions leading to the aggregation of denatured β-LG (Oldfield, Singh, Taylor, & Pearce, 2000). Initially, hydrophobic aggregates and disulfide-linked aggregates of β-LG

seem to form concurrently during heating. However, with continued heating, the hydrophobic aggregates undergo thiol-disulfide interchange reactions. As temperatures surpass 70 °C, where hydrophobic interactions between protein molecules weaken (Oldfield et al., 2000), the whey protein aggregates could potentially break down into their unfolded monomeric components. Subsequently, these monomeric proteins may engage in thiol-disulfide interchange reactions. Alternatively, the hydrophobic interactions within the aggregate could persist, and intermolecular disulfide bonds may form either within the aggregate or between β-LG aggregates. Therefore, aggregates containing disulfide bonds should not be considered exclusive of hydrophobic interactions. Moreover, steric effects appear to be significant in influencing disulfide bond formation during the heating of whey proteins (Oldfield et al., 2000). The aggregates of β-LG extend from the surface of the micelles, creating a physical barrier that hinders further association of β-LG. Additionally, the reactive sulphydryl groups within these aggregates are likely enclosed, rendering them inaccessible for reactions involving sulphydryl–disulfide exchange with micellar κ-CN. High ionic strength mitigates electrostatic repulsion, thereby facilitating protein aggregation as proteins can come together more easily and in a wider range of orientations, leading to an increased formation of disulfide bonds. Consequently, the involvement of thiol groups in forming disulfide bonds seems to be influenced by their initial position within the protein's native structure, with cysteines on the surface being more likely to participate than those located internally. This suggests that the specific location of cysteine residues within the original tertiary structure of a protein plays a crucial role in determining their availability and subsequent participation in disulfide bond formation, despite protein unfolding during heat treatment (Livney, Verespej, & Dalgleish, 2003; Lowe et al., 2004).

The behaviour of milk proteins during heating, particularly the interaction between κ-CN and β-LG complexes with casein micelles, is highly influenced by the pH of the milk. When milk is heated above a pH of 6.9, the κ-CN/β-LG complexes tend to dissociate from the micelle, whereas at a pH below 6.7, these complexes stay attached and stabilize the micelle thereby reducing the likelihood of casein dissociating. This detachment of κ-CN upon heating is associated with the creation of serum aggregates, though it is still not clear whether these aggregates result from or cause κ-CN dissociation. A model proposed by Vasbinder & De Kruif (2003), delves into the pH-dependent dynamics of whey protein association with casein micelles. This model elucidates that heating milk at a pH greater

than 6.6 results in only partial coverage of casein micelles by denatured whey proteins, whereas at pH levels below 6.6, a more comprehensive attachment occurs. Notably, at a pH of 6.55, micelles receive a fairly uniform coverage, yet altering the pH beyond this point leads to uneven coating of the micelles which indicates how subtle pH adjustments can significantly influence the protein interactions and the micelle's structural integrity. Research has further revealed that the distribution of complexes between soluble and micellar phases is contingent upon the milk pH during heating. Denatured whey proteins are more inclined to adhere to the casein micelles in a low pH environment, forming more stable structures. In contrast, heating milk at a higher pH facilitates the formation of more soluble complexes, shifting the balance towards a different set of interactions (Donato & Dalgleish, 2006; Vasbinder & De Kruif, 2003). The final composition of heated milk is a complex blend of native whey proteins, whey protein aggregates, and casein micelles, which are variously covered with denatured whey proteins. This composition is predominantly dictated by the pH level, the temperature, and the duration of the heat treatment. According to the findings of Anema & Li (2003b), at a pH of 6.5, a substantial portion (75-80%) of denatured whey proteins associates with the micelles, a figure that diminishes to about 30% at a pH of 6.7. The casein micelle size also reacts to these pH changes, significantly increasing by approximately 25-30 nm at pH 6.55, while only expanding by about 5-10 nm at pH 6.7 when subjected to heating at 90°C for 30 minutes. The underlying reasons for these size variations, whether due to whey protein association or partial casein micelle aggregation, remain a topic of ongoing investigation (Anema & Li, 2003a; Singh, 2004). This nuanced understanding underscores the critical role of pH in determining the structural and compositional outcomes of milk proteins upon heating, highlighting the intricate balance between protein stability and aggregation dynamics.

2.5.2 Dissociation of caseins

The underlying mechanisms for the dissociation of caseins with varying temperatures are believed to be primarily due to shifts in the electrical charge of the casein proteins and the dynamic state and composition changes of CCP. These elements collectively contribute to the overall stability and reaction of casein in milk systems under heat treatment, highlighting the complex interplay between temperature, pH, and milk composition in affecting the solubilization of caseins. The tendency for caseins to dissociate from micelles increases concurrently with increases in the milk pH and

temperature. Studies have shown that the order of dissociation follows κ-CN>β-CN> α_S-CN under specific pH and temperature settings (Anema & Klostermeyer, 1997; Law & Leaver, 1997). A notable minimal dissociation of κ-CN from micelles occurs at a pH of 6.6 during heating, whereas about 50% becomes soluble upon heating to 120 °C at pH 7.1. This κ-CN dissociation can be partially reversed by adjusting the pH (Singh & Latham, 1993). The majority of casein dissociation occurs rapidly at the onset of the heat treatment, with minimal further separation observed during extended periods of heating (Anema & Klostermeyer, 1997). Micelles from which κ-casein has been removed are considered to have significantly lower stability compared to those with intact κ-CN (Walstra, 1990). Therefore, the removal of the steric protection κ-CN offers leads to the increased aggregation of casein micelles (O'connell & Fox, 2003).

The integrity of casein micelle structure is predominantly upheld through CCP linkages and hydrophobic forces, alongside less significant electrostatic and van der Waals interactions. Alterations in temperature and pH may modify these interactions, potentially leading to the disassembly of micelles (Anema & Klostermeyer, 1997). The charge of κ-CN therefore plays a crucial role in maintaining its association with micelles (Singh & Creamer, 1991). Dissociation of κ-CN at elevated temperatures (≥90 °C) and at higher pH levels results from the irreversible changes to the protein charge due to the impact of heat. Nevertheless, the total charge of the dissociated caseins does not undergo notable changes, suggesting that changes in the charge distribution of the proteins do not induce their dissociation (Anema & Klostermeyer, 1997). Dissociation of casein micelles may be due to changes in the properties of CCP under high pH and temperature conditions, rendering it less effective at preserving micellar integrity. However, further empirical evidence necessary to substantiate this theory, such as a detailed analysis of the compositional and structural modifications of CCP in milk under varying temperatures and pH levels, remains insufficient (Anema & Klostermeyer, 1997). Horne (1998) suggested that elevating the pH results in the transformation of SerP into forms with double negative charges, which fail to stabilize CCP. Given the role of CCP in micelle stability, its dissolution results in the subsequent release of caseins into the solution (Anema & Klostermeyer, 1997).

2.5.3 Other changes in milk systems

The decrease in milk pH as the temperature exceeds 120 °C for extended periods is attributed to several chemical reactions. These include the isomerization of lactose into lactulose, the breakdown of lactose into epilactose and formic acid, and the dephosphorylation of casein (van Boekel, 1998). Casein dephosphorylation occurs either through the hydrolysis of phosphoserine, resulting in phosphate production, or through β-elimination, which generates dehydroalanine (DHA) and phosphates (Singh, 2004; van Boekel, 1999). These processes collectively contribute to the alteration of milk acidity with prolonged heating.

The Maillard reaction, a process triggered by heating at elevated temperatures (>120 °C) for extended durations (O'connell & Fox, 2003), involves several key stages. Initially, lactose and amino groups (from lysine) undergo condensation to form Schiff base compounds, which then rearrange into an Amadori product specifically, lactulosyllysine through Amadori rearrangements. Subsequent decomposition of the Amadori product yields a variety of compounds including formic acid and lysylpyrraline and the reaction culminates in the creation of melanoidins during the final stage. The outcomes of these reactions include diminished nutritional value, the emergence of flavour compounds, the formation of compounds with antioxidative and antibacterial properties, the polymerization of milk proteins, and a noticeable brown colouring attributed to the melanoidins. Moreover, the early reaction phase involving lysine degradation contributes to an increased negative charge on casein micelles (van Boekel, 1998).

During thermal processing, the breakdown of proteins, known as proteolysis, is a notable alteration in milk proteins (Morales & Jiménez-Pérez, 1998). This process is facilitated by the absence of secondary structures in caseins, which are proline-rich and adopt an open, flexible conformation, making them susceptible to heat-induced proteolysis. Such changes within milk systems due to proteolysis include the augmentation of polar groups such as NH^{3+} and COO^{-}, a reduction in the molecular weight of peptide chains, and alterations in the process of aggregation of casein molecules (Gaucheron, Mollé, & Pannetier, 2001).

It has been revealed that, subjecting milk to high-temperature heat treatment (>120 ℃) not only enlarges the casein micelles but also leads to an increase in the presence of

protein particles that are smaller than the micelles (El-Din & Aoki, 1993). Prolonged exposure to ultra-high temperatures can further alter the covalent structures of proteins, including removing phosphate groups from phosphoserine residues, and breaking down proteins into peptides and amino acids (Singh & Latham, 1993). These processes yield particles smaller than the original casein micelles. The growth in particle size at elevated temperatures is likely attributed to the polymerization of casein and whey proteins through condensation reactions, including those similar to Maillard reactions and the formation of lysinoalanine, with lactose playing a crucial role in casein polymerization upon heating. The heating process also promotes the polymerization of caseins through the formation of lysinoalanine which results from the β-elimination of phosphoseryl residues (El-Din & Aoki, 1993; Gaucheron et al., 2001). Additionally, deamidation occurs, releasing ammonia (van Boekel, 1999).

2.6 Reconstituted milk protein products

Milk is well known as a valuable source of proteins and other nutrients; however, many global regions lack adequate climate and geographic conditions to produce their own milk. In addition, other regions encounter irregular yearly production and/or face difficulties in fresh milk transportation. As a result, milk producers in these regions optimise their production using reconstituted milk to accommodate growing demands. Moreover, greater consumer awareness of importance of high protein diets for weight loss, muscle building and prevention of sarcopenia has led to an increased demand for high protein processed foods. To obtain the desired levels of proteins, fat, carbohydrates and sensory attributes, manufacturers combine other ingredients with high protein dairy powders (Baxter, Dimler, & Rangavajala, 2011). Reconstituted skim milk powder, whole milk powder, milk protein concentrates and casein and whey protein concentrate powders are used in range of dairy protein-based beverages, which are required to contain high protein levels without compromising their stability and quality. In addition to high protein dairy beverages, there are several other applications of reconstituted milk proteins including infant formula, medicinal and nutritional speciality products, cheese, yoghurt, confectionery, ice cream and high protein bars. The unique nutritional, sensorial, functional and physical properties have contributed to an immense potential to tailor the various attributes and the scope for manufacturing a wide array of food products.

2.6.1 Milk protein concentrates

Milk protein concentrates (MPC) have a wide range of applications including their addition to milk or cheese formulations to increase the protein content or yield of the final product, and to enhance the textural characteristics of yogurts. They have also found growing popularity in nutritional drinks due to their low lactose content and similar ratio of whey to casein proteins rendering a high protein content (5-10%).

MPCs are primarily produced using membrane technology which results in a final protein content in the range 50-85%. As shown schematically in Figure 2.3, MPC manufacturing involves the concentration of skim milk by ultrafiltration (UF) to increase the protein content and the removal of lactose and salts. Some calcium, magnesium, phosphate and citrate associated with the casein micelles are retained in the concentrate. Diafiltration (DF) is then usually applied to obtain >70% protein on a dry powder basis after which the retentate is evaporated to remove some more water prior to spray drying of the final product.

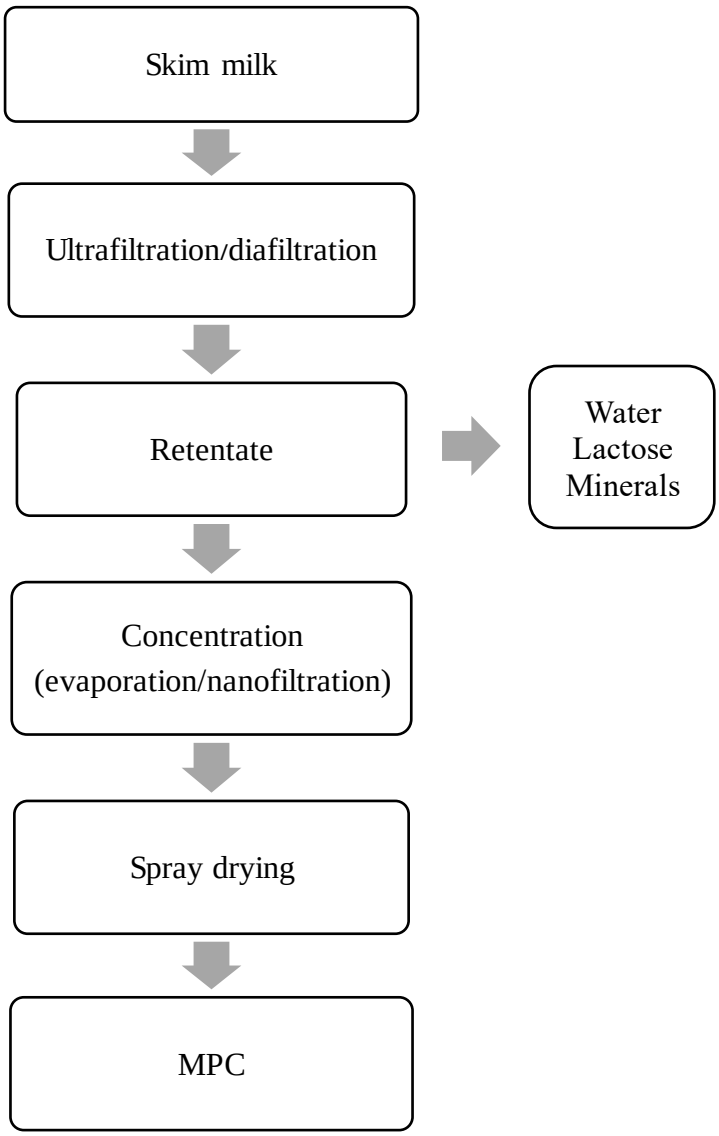

Figure 2.3 Flowchart of MPC powder manufacturing adapted from Sharma (2021)

As the process involves no preheating, except pasteurisation, whey proteins remain largely in their native state (Singh, 2007). The volume distribution and average diameter of casein micelles have been reported to decrease upon UF processing, mainly due to changes in calcium and phosphate levels in milk (Srilaorkul, Ozimek, Ooraikul, Hadziyev, & Wolfe, 1991). In contrast, McKenna (2000) observed little change in particle size during the early stages of UF, however, after DF the average micelle size increased significantly. Imaging studies using scanning electron microscopy showed the progressive swelling of casein micelles and the formation of non-micellar materials. After evaporation, the micelles were observed to pack together resulting in aggregation linked

via non-micellar material. In addition, the loss of serum calcium and phosphate during UF and DF processes results in the dissolution of colloidal calcium phosphate and this leads to a loosening of the casein micelle structure resulting in their swelling. Subsequent evaporation results in an increase of micellar aggregation involving non-micellar material and/or dissociated casein micelles. Spray drying was suggested to induce further interactions among proteins; however, the nature of these interactions is still unknown (Singh, 2007). As a result of the overall processes involved in MPC production, a comparatively high level of dissociated caseins would be expected after its reconstitution.

Rehydration of MPC powders is a time-consuming process that is significantly affected by several factors such as protein and mineral content, skim milk heat treatment, spray drying temperature, powder storage conditions (time and temperature) and the MPC rehydration process (McCarthy, Kelly, Maher, & Fenelon, 2014). The powder particle dissolution process is a rate limiting step during rehydration due to the casein micelle fusion caused by hydrophobic interactions (Horne, 1998). Reconstitution of MPC powders is usually performed at 50 °C for optimum dissolution in several product manufacturing applications including cheese and beverages (Havea, 2006).

Since processing creates significant differences in the composition of non-protein constituents of MPC and skim milk powder (SMP), reconstituted MPC contains more protein per unit of total solids than reconstituted SMP (Deeth & Hartanto, 2009). Reconstituted MPC presents an altered mineral environment as compared to reconstituted SMP as a result of the UF and DF steps used to concentrate the milk proteins. Therefore, MPC contains more calcium than SMP, however, it contains less calcium per unit of protein (Kelly, 2011).

2.6.2 Composition and structure of MPC

MPCs are complete milk proteins, containing both caseins and whey proteins with the typical composition of MPC80 presented in Table 2.5. The mineral content of these products may differ due to variations in skim milk and the temperatures used during UF and DF processes as reported by Augustin, Oilver & Hemar (2011).

Table 2.5 Typical composition of MPC80*

Component	MPC80
Protein % dry basis	79.4
True protein (%)	78.4
Non-protein nitrogen (%)	1.6
Fat (%)	1.7
Moisture (%)	5.0
Lactose (%)	5.5
Ash (%)	7.8
Calcium (mg/100g)	2450-2550
Phosphorous (mg/100g)	1500-1700
Potassium (mg/100g)	250-300
Sodium (mg/100g)	120-150

*Source: Novak (1992)

2.6.3 Functional properties of MPC

Milk protein concentrates are integral to various food formulations, offering unique benefits due to their reduced lactose and mineral content (Martin, Williams, & Dunstan, 2007). However, the functional effectiveness of MPCs, including aspects such as emulsification, foaming, viscosity, and gel formation, is significantly influenced by their solubility which is a critical factor for optimal performance in food products. This solubility is not only vital for maintaining product stability but also affects other properties such as moisture retention and stability under heat, acidic conditions, and freeze-thaw cycles (Corredig, Nair, Li, Eshpari, & Zhao, 2019). The utilization of MPCs, especially in enhancing whey protein levels in specific formulations, faces limitations due to the denaturation of whey proteins at high processing temperatures. Such heat-induced denaturation leads to the formation of bonds between whey proteins and caseins resulting in defects, diminished functionality, and other issues including precipitation and flocculation. These problems can adversely affect crucial characteristics of food products, including their freezing point and viscosity. Moreover, the solubility challenges of MPCs particularly impact the dairy beverage industry, where poor solubility can result in sedimentation and reduced shelf-life. Processing parameters, such as spray drying conditions, atomizer and dryer technology, and storage conditions (including temperature,

humidity, and time), significantly influence MPC solubility. Additionally, rehydration conditions including water temperature and time, are pivotal for effective MPC reconstitution. Increasing the temperature to around 50°C during reconstitution generally enhances solubility by causing proteins to unfold and disrupt their secondary and tertiary structures (Fang, Selomulya, Ainsworth, Palmer, & Chen, 2011). However, higher temperatures may lead to protein aggregation and precipitation due to hydrophobic and covalent interactions, thereby reducing solubility. Despite the advantages of using high temperatures for sterilization and ultra-high temperature (UHT) treatment to extend shelf-life, these processes can compromise the solubility of MPCs over time (Havea, 2006).

The rate-limiting phase in rehydrating powder particles is their dissolution, rather than the initial wetting or deagglomeration (Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2009). The dissolution kinetics of these powders can be altered by storage-induced effects, potentially due to micellar interactions over time (Anema, Pinder, Hunter, & Hemar, 2006; Havea, 2006). The powder stability, particularly for those high in protein, is significantly influenced by the moisture content and the temperatures at which they are stored (Richard et al., 2013). Factors such as the pH and temperature of the solution, the ionic concentration, and the type of powder being used all play roles in affecting the solubility of MPC powders. Charge and electrostatic interactions of proteins impacted by the pH, are crucial for solubility. At a neutral pH of 7, MPCs with protein content up to 70% show high solubility in water, but those with higher protein concentrations exhibit poor solubility at room temperature as reported by Augustin & Clarke (1990). The solubility of MPC powders improves with elevated reconstitution temperatures, with α- and β-CN constituting most of the insoluble elements of the powder (Havea, 2006). Moreover, the thermal stability of MPC suspensions tends to decrease as the protein concentration increases (Crowley et al., 2014) Further details regarding functional properties of MPCs is discussed in Section 2.7.

MPCs also possess an emulsifying capacity (EC) that is highly beneficial across various sectors including meat and canning industries. Studies have demonstrated that the EC diminishes as the level of heat treatment during MPC production increases (Babella, 1989). These findings also indicated that a higher ratio of calcium to total minerals in MPC leads to reduced solubility and lower EC. Although adjusting the calcium to total mineral ratio does not impact the ability of MPC to bind water, it lowers the solubility

and EC. In contrast, elevating the ratios of potassium or sodium significantly enhances the water-binding capacity of MPC while diminishing the EC, but without altering the heat stability or solubility (Babella, 1989).

2.7 High protein dairy beverages

The rising popularity of ready-to-drink (RTD) high protein beverages highlights the growing consumer demand for convenient options to boost protein consumption. Dairy proteins, notably from milk and whey, are the most popular choices for these RTD formulations, particularly those that contain at least 15 g of protein per serving (Oltman, Lopetcharat, Bastian, & Drake, 2015). The market today highlights a diverse range of protein contents in these beverages, from 10-40 g per serving, a variability that often stems from the wide range of serving sizes which fluctuate between 236 and 591 mL (Vogel, Carter, Cheng, Barbano & Drake, 2021). In response to the demand for higher protein contents, manufacturers utilize a wide array of dried milk-derived protein ingredients to create these nutrient-rich drinks. The selection spans from traditional options like non-fat dry milk, MPC and isolates, to whey protein concentrates and isolates. Moreover, the field continues to innovate with the introduction of new ingredients such as micellar casein concentrate (MCC) and milk serum protein isolate (SPI), the latter essentially being a milk-derived whey protein concentrate (Liu, Toro-Gipson, & Drake, 2021). This evolving landscape of RTD protein beverages, however, does not come without its challenges. As manufacturers strive to increase the protein concentrations to meet consumer expectations, they face intricate challenges in formulation and production processes. These include ensuring thermal stability and maintaining appealing flavours, both of which become increasingly difficult as protein levels rise. Despite these obstacles, efforts to cater to the growing consumer interest in protein-enriched beverages are evident, reflecting a commitment to innovation and quality in meeting nutritional needs.

2.7.1 Processing pH

Ready-to-drink beverages are classified into two distinct categories based on their pH levels: neutral pH beverages and acidified beverages. Beverages that maintain a neutral to low acidity, range from a pH of 4.6 to 7.5 (Etzel, 2004), whereas acidified beverages are characterized by a lower pH range of 2.5 to 3.0. This pH differentiation is crucial as

it dictates the required thermal treatment to ensure microbial safety, with beverages having a pH below 4.6 being less heat-resistant and more susceptible to bacterial degradation (Rodrigo, Sampedro, Silva, Palop, & Martínez, 2010). As a result, the thermal processing techniques vary significantly between these two types of beverages. Acidic RTD options are typically treated using the "hot fill" method, where the liquid is heated to at least 83 °C (with a preference for 90 to 95 °C), poured into containers, and then inverted while still hot to sterilize the interior surface (Vogel III, Carter, Cheng, Barbano, & Drake, 2021). Conversely, shelf-stable beverages with a neutral pH undergo more intensive sterilization processes such as retort processing at temperatures of 115-125 °C for 10 to 40 minutes or UHT processing, which exposes the beverage to 135-150 °C for a few seconds. After these treatments, beverages are aseptically filled into pre-sterilized containers. Ultrapasteurization is an alternative method that heats the beverage to 138 °C for a minimum of 2 seconds, followed by refrigeration to achieve an extended shelf-life. The suitability of protein types for inclusion in RTD beverages is also influenced by the product pH and the chosen thermal treatment. Acidic conditions prevent the use of casein-containing milk protein ingredients due to their propensity to precipitate at a pH lower than 4.6. However, whey proteins can dissolve effectively in such acidic environments. In contrast, at neutral pH levels, caseins demonstrate superior heat stability compared to whey proteins. Therefore, in these neutral pH beverage formulations, casein-rich ingredients (casein to whey protein ratio 80:20 or higher) are commonly used due to their superior heat stability (Singh et al., 2022). MPC, milk protein isolates and micellar casein concentrates are the most common protein-based ingredients used in neutral pH dairy beverages (O'Mahony & Fox, 2014).

2.7.2 Heat stability

Neutral pH or low acid high protein dairy beverages are subjected to intense heat treatments to extend their shelf-life. Therefore, heat stability is an essential consideration when selecting protein ingredients. The heat stability of reconstituted MPC plays a crucial role in maintaining the desired functional characteristics in food products, including solubility, emulsification, foaming capacity, viscosity, water-holding capacity, gel formation, freeze-thaw resistance, and stability in acidic conditions (Anema et al., 2006; Corredig et al., 2019). These properties are closely interconnected and significantly influence the overall performance of food systems. Therefore, assessing the heat stability

of MPCs is vital to ensure uniform processing and for preserving the quality of the final product, while avoiding the negative impacts that may arise from thermal destabilization.

The stability of MPC dispersions when exposed to heat can be influenced by several factors. These factors include the amount of protein present in the MPC powders, the specific membrane filtration methods employed during powder production (such as DF or nanofiltration), the type of water used for reconstitution (either reverse osmosis or treated water, which vary in ionic strength), and the concentration of protein in the final formulation (Lin, Kelly, O'Mahony, & Guinee, 2018). Crowley et al., (2015) examined the heat coagulation time (HCT) of MPC dispersions using powders with protein concentrations ranging from 35% to 90% that were prepared at a concentration of 3.5% (w/w). The experiments were performed over a pH range of 6.3 to 7.3 and at a temperature of 140 °C. A reduction in HCT for dispersions composed of MPC with protein contents exceeding 80% was observed and this phenomenon was attributed to the augmented activity of calcium ions within the solution. Further investigations by Sunkesula, Kommineni, Meletharayil, Marella, & Metzger (2021) revealed that dispersions containing 10% MPC, specifically those derived from 80% protein MPC powders, demonstrated a distinctive HCT pH profile divergent from that of traditional skim milk formulations. The most pronounced HCT for the 10% protein dispersion from MPC80 was recorded at a pH of 6.9 with a notable decrease at pH 7.1. The augmented HCT at pH 6.9 was ascribed to the increased calcium ion activity, which likely facilitated the interaction between whey protein and casein on the surface of the micelles, thus generating a steric obstruction around the casein micelles. In contrast, at pH 7.1, it was observed that the interaction between κ-CN and whey protein occurs within the serum phase, resulting in a lower HCT. In addition, reduced mineral content (30% less calcium) resulted in an increase in HCT at pH 6.9 compared to the standard control. This suggests that lowering colloidal calcium content during the production of MPC can enhance the heat stability of MPC dispersions with reduced calcium contents (Sunkesula et al., 2021). Moreover, incorporating calcium-chelating salts such as sodium hexametaphosphate, was reported to enhance the heat stability of MPC protein dispersions.

Ingredients enriched with casein, e.g., micellar casein concentrate (MCC), may demonstrate heat stability profiles that differ from those of skim milk or MPC dispersions. When evaluating MCC dispersions against MPC dispersions at equivalent protein

concentrations, it is observed that MCC dispersions possess enhanced heat stability. A significant factor influencing this increased stability is the increased ratio of casein to whey protein (Renhe & Corredig, 2018).

2.7.3 Solubility

Ensuring that milk proteins are properly hydrated prior to thermal processing is crucial for unlocking their full functional potential, including their capabilities related to emulsification and heat stability (Mimouni et al., 2009). Inadequate hydration of protein ingredients can adversely affect the functional qualities of the protein, potentially impacting the sensory attributes and shelf-life of the final beverage products. This is typically manifested by significant issues such as a phase separation, grainy or chalky textures and altered viscosities.

The rehydration of dairy powders follows five essential phases: wettability, swelling, sinkability, dispersibility, and dissolution (Crowley, 2016). Initially, wettability pertains to the capacity of the powder to absorb water which is then followed by the expansion of the powder particles in the swelling phase. Sinkability describes how these expanded particles descend into the liquid environment. The next phase is related to the capacity of the powder to either spread throughout the water (disperse) or to begin to dissolve. The concluding phase of dissolution focuses on the breakdown and separation of the powder particles into finer particles. Figure 2.4 illustrates the rehydration process which does not follow a particular sequence; several phases might overlap or occur concurrently.

The insolubility of MPC powders poses a significant challenge in the production of dairy protein beverages. When MPC powders (MPC32 to MPC85) are freshly made, they exhibit solubility levels greater than 95% (Gazi & Huppertz, 2015). A negative relationship between the solubility of MPC and the duration and temperature of storage was reported, particularly for MPC variants with protein concentrations of 50% or higher (Gazi & Huppertz, 2015). This insolubility issue is partly attributed to the micellar fraction; however, an increase in the soluble casein fraction can enhance the solubility of MPC powders. To improve the solubility of casein in MPC powders, various methods have been developed. One such method involves reducing minerals through the injection of CO_2, which has been shown to enhance the solubility of MPC80 powders (Marella, Salunke, Biswas, Kommineni, & Metzger, 2015). Another approach is the ion exchange

treatment of the UF retentate resulting in a calcium-reduced MPC powder, thereby improving its solubility (Xu et al., 2016). The use of membrane filtration at acidic pH levels, and the addition of calcium sequestering salts (CSS) are some other methods to improve MPC solubility (Carr & Golding, 2016). These approaches aim to diminish the content of CCP while augmenting the proportion of non-micellar casein, thus improving the thermal stability of the product (Carr & Golding, 2016).

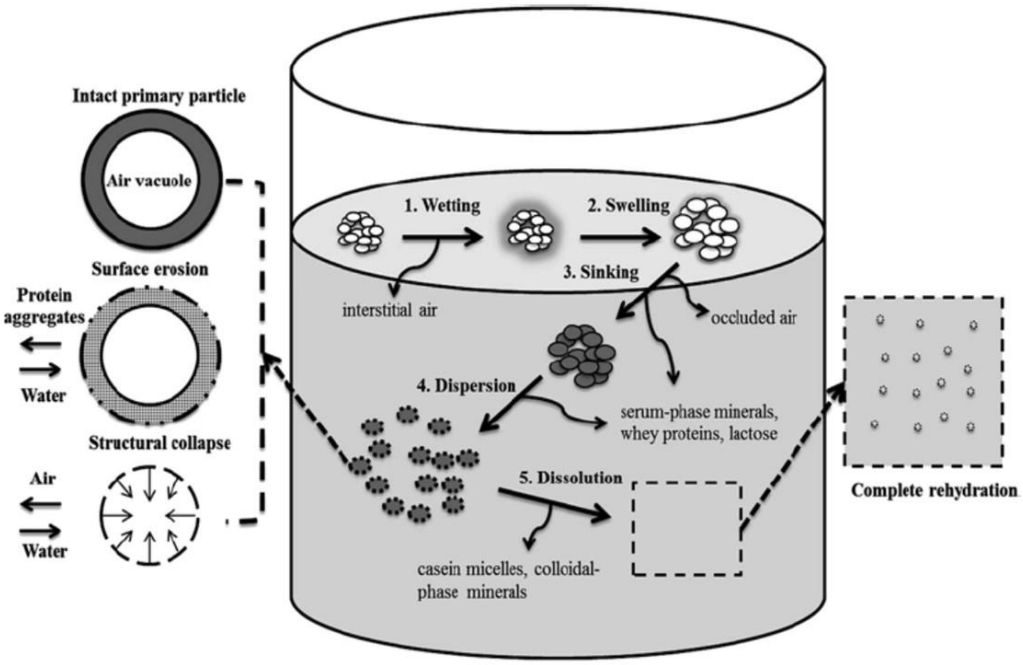

Figure 2.4 Illustration of the rehydration of high protein dairy powders from Crowley (2016)

Calcium sequestering salts function by binding to multivalent metal ions, indirectly leading to the demineralization of casein micelles. Nevertheless, the use of CSS in excessive amounts can adversely affect thermal stability by removing essential CCP levels, thereby compromising the structural integrity of the micelles (Mizuno & Lucey, 2005). In the dairy industry, phosphate and citrate are frequently utilized as thermal stabilizers, with their concentrations typically not exceeding 40 mmol per kilogram of skim milk solids (Augustin & Clarke, 1990). Table 2.6 summarises some of the attributes of casein micelles and mechanism of CSS in relation to these attributes.

Table 2.6 Impact of different calcium sequestering salts on casein micelles*

Characteristics	Impact of CSS	CSS level
Calcium chelation ability	Long-chain phosphates > tri-polyphosphates > pyrophosphate (triphosphates and di-phosphates) > citrate > orthophosphate	⬆
Casein fraction solubilisation	Solubilization sequence - $\alpha_{s1} > \beta > \alpha_{s2}$ and κ-CN (proportion of individual soluble caseins remains unchanged by the type of CSS)	⬆
CSS and calcium complexes	Phosphates – insoluble calcium phosphate complexes Citrates – soluble calcium citrate complexes	-
Dispersion of caseins	Disodium uridine phosphate < disodium phosphate < trisodium citrate < sodium phytate < sodium hexametaphosphate	⬆
Emulsion droplet size	Tripolyphosphates > pyrophosphates > polyphosphates > citrates ≈ orthophosphates ≈ sodium aluminium phosphates	-
Hydration of caseins	Phosphates: increasing chain length of sodium phosphates increase casein hydration Citrates: lower than ortho- and pyro-phosphates	⬆

*Data taken from Deshwal, Gómez-Mascaraque, Fenelon, & Huppertz (2023)

2.7.4 Hydrocolloid additives

Hydrocolloids are components that form colloidal systems within water owing to the hydrophilic segments of their molecular structure (Jafari, Beheshti, & Assadpour, 2013). These substances are characterized as long-chain biopolymers that can be easily dispersed or dissolved, either partially or completely, and tend to expand upon encountering water. Various biopolymers, encompassing both polysaccharides and proteins, have been utilized in dairy product formulations to enhance texture, rheological behaviour, physicochemical characteristics, and sensory qualities, aiming to fulfill consumer preferences (Nishinari, 2021). The use of hydrocolloids in the dairy sector relies on two primary functions: firstly, they aid in the formation of milk gels by providing a structured network to liquid mixtures, and secondly, they are capable of extending the shelf-life of products (Yousefi & Jafari, 2019). The physical structure of gels is primarily due to hydrogen bonding, hydrophobic interactions, and cation-dependent cross-linking, leading to their classification as "physical gels" (Munarin, Tanzi, & Petrini, 2012). Numerous studies have explored the gelation process involving the integration of casein micelles

with hydrocolloids in milk, focusing on their impact on the physical and rheological properties of the milk. Among the hydrocolloids frequently employed for gel formation are κ-carrageenan, gelatin, and pectin, (Pang, Deeth, Sopade, Sharma, & Bansal, 2014). Hydrocolloids have the ability to extend the shelf-life of dairy products by minimizing wheying-off, suspending dispersed particles, and preventing the aggregation of protein micelles (Sahan, Yasar, & Hayaloglu, 2008). The key characteristics of the hydrocolloids most widely used in dairy systems are summarised in Table 2.7. In the following section, a detailed examination of the use of carrageenans in dairy beverages is presented, highlighting its relevance to the current study.

Table 2.7 Key properties of commonly utilized hydrocolloids*

Hydrocolloid	Characteristics
Carrageenan	• negatively charged • κ-carrageenan: coil to helix transition at 40-60 °C; brittle, strong and opaque gels; ~25% sulphated • γ-carrageenan: coil to helix transition at 40-60 °C; soft, elastic, and transparent gels; ~32% sulphated • ι-carrageenan: coil form; ~ 35% sulphated; used for thickening
CMC (carboxymethyl cellulose)	• negatively charged • acts as a stabilizing agent • soluble in cold and hot water • improves viscosity
Pectin	• negatively charged • high-methoxyl pectin (HMP): gels at 65-70% sugar, pH 3.2-3.5 and 0.2-1.5% pectin • Low-methoxyl pectin (LMP): gels at 10-70% sugar, pH 2.6-7 and 15 mg/g calcium
Loust bean gum	• non-ionic • no gel formation • linear polysaccharides • soluble in cold and hot water • improves viscosity • used as a fat replacement • heat and pH: synergistic action with carrageenan and xanthan delivering a creamy mouthfeel in ice cream, cream-cheese
Inulin	• non-ionic • fructooligosaccharide (FOS, 3–8 fructose) and high-performance inulin (11–65 fructose) • sugar and fat replacement • forms gels containing insoluble microstructures

Hydrocolloid	Characteristics
Tragacanth	• negatively charged • thickening agent, emulsifier, suspending agent, fat replacement • enhances viscosity and steric repulsion and in a protein solution
β-glucan	• non-ionic • improves viscosity • contains high dietary fibres • thickening and non-caloric stabilizing agent in ice-cream, cheese

*adapted from Yousefi & Jafari (2019)

2.7.5 Carrageenan

Carrageenan, a linear, sulphated polysaccharide obtained from different species of edible red seaweed within the *Rhodophyceae* family, is extensively utilized as a gelling agent, stabilizer, or thickener in various food products, pharmaceuticals, and cosmetic formulations (Wurm, Pham, & Bechtold, 2019). The molecular composition of carrageenan consists of repeating disaccharide sequences, alternating between D-galactose and 3,6-anhydro-galactose (3,6-AG) units, which are connected through α-1,4 and β-1,3 glycosidic bonds (Li et al., 2019). Carrageenans are classified by different types, however κ-carrageenan and ι-carrageenan are the most common types used in the food industry (Bui, Nguyen, Nicolai, & Renou, 2019).

Carrageenan can adopt either a random coil or a helical structure, with the differences in these structures facilitating their adsorption to proteins (Schefer, Usov, & Mezzenga, 2015). The λ-carrageenan type readily binds to milk proteins at all temperatures due to its existence in a highly charged coiled form, which contains three sulfate groups per disaccharide unit (Stone & Nickerson, 2012). Conversely, κ- and ι-carrageenan, which have one and two sulfate groups per disaccharide respectively, shift to helical structures as temperatures drop from 60 to 35 °C. These biopolymers have been observed to attach to milk proteins in their helical state (Arltoft, Ipsen, Madsen, & De Vries, 2007). The transformation from coil to helix increases the charge density by bringing the charges closer together, facilitating the binding of κ- and ι-carrageenan with milk proteins at temperatures below 60 °C. Moreover, the formation of helical structures is associated with the gelation of carrageenan (Banerjee & Bhattacharya, 2012). The coil-to-helix transition of carrageenan and its implications for gel formation is depicted in Figure 2.5 (Liu, Zhan, Wan, Wang, & Wang, 2015).

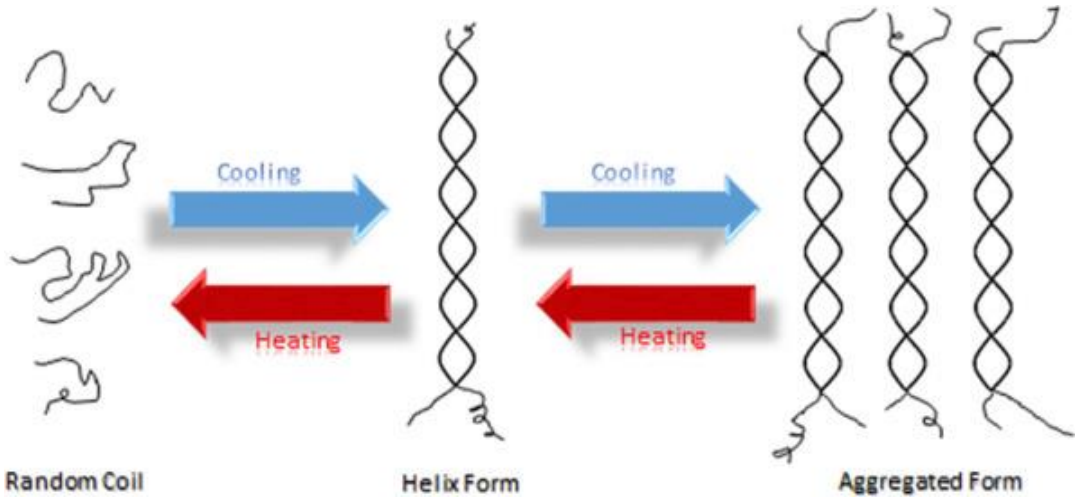

Figure 2.5 Representation of the gelling mechanism of carrageenan modified from Yousefi & Jafari, (2019), originally taken from Liu et al., (2015)

At ~pH 6.8, electrostatic interactions take place between κ-carrageenan and κ-CN on the surface of casein micelles. Importantly, the section of κ-CN that spans amino acids 97 to 112 is crucial for these interactions due to its significant positive charge, which can form electrostatic links with the negatively charged sulphate groups found in κ-carrageenan (Langendorff et al., 1999). These linear polymers in κ-CN are characterized by varying levels of sulphate half-ester groups that impart a negative charge, influencing their characteristics and functionalities. Additionally, cations including calcium can act as connectors between protein carboxyl groups in milk and the sulphate groups of carrageenan (Drohan, Tziboula, McNulty, & Horne, 1997; Spagnuolo, Dalgleish, Goff, & Morris, 2005). A schematic illustration depicting the reactivity between κ-carrageenan and κ-CN is shown in Figure 2.6.

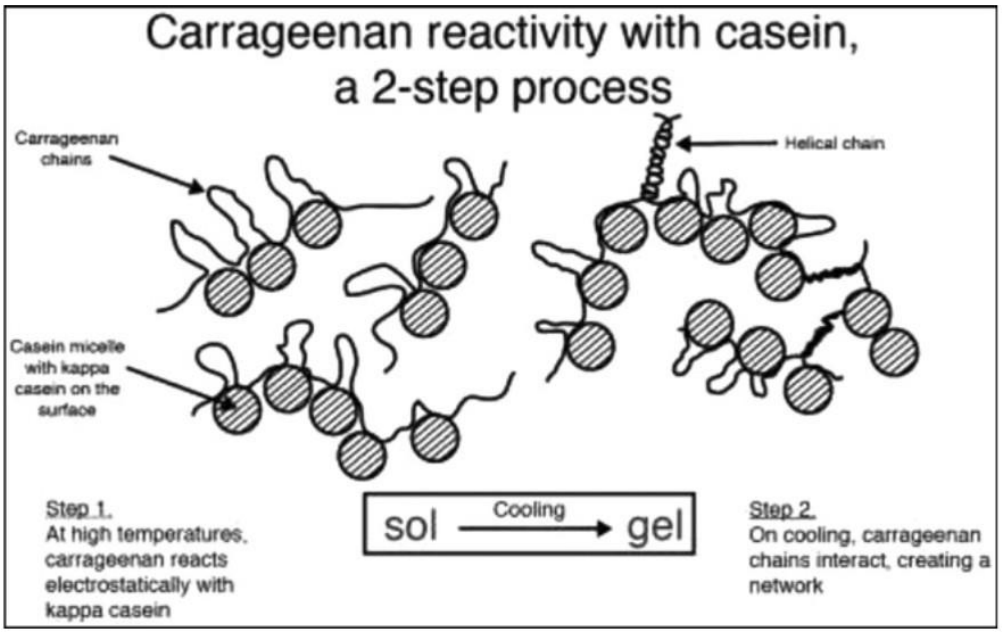

Figure 2.6 Illustration of interaction between κ-carrageenan and casein from Blakemore, Davis, Hroncich, & Vurma (2014)

2.8 Shear induced milk protein interactions

During the processing of dairy fluids, diverse physical phenomena manifest, including fluid dynamics, thermal convection, thermal conduction, and molecular diffusion. Both dairy and plant-based substances, irrespective of their state as either liquid or solid, demonstrate a proclivity for movement under the influence of applied forces over temporal intervals. This force, represented as a stress vector (σ), may induce deformation in the system, quantified as strain (γ), with its time derivative defining the strain rate ($\dot{\gamma}$) (Walstra, 2003):

$$\sigma = \eta \frac{d\gamma}{dt} = \eta\dot{\gamma} \quad (1)$$

When applied to a fluid, as in the case of pumping or homogenisation, stress results in laminar or turbulent flow with each exhibiting distinct characteristics. The transition from laminar to turbulent flow involves chaotic streamlines, creating vortices that enhance mixing and kinetic energy dissipation (Williams, Rosenblat, & Stuart, 1969). Understanding the behaviour of proteins in this dynamic environment is crucial, especially considering the marginal stability of their folded conformations under optimal conditions. The delicate balance between the stability and instability of native proteins

involves intricate interactions and compensations, including conformational degrees of freedom, water dynamics, and solvent interactions. The small free energy gap governing the transition from the native to denatured state of proteins is influenced by entropic and enthalpic contributions, subsequently impacting protein stability and folding (Dickinson & McClements, 1995). In addition, the solvent, which is often a mixture of water and cosolvents, plays a significant role in inducing unfolding or folding, leading to changes in critical physical parameters that delineate the phase boundaries between native and denatured states. Other parameters, including the concentration of proteins and solids, the pH of the system, chemical additives such as chelators, and the presence of additional components like hydrocolloids and flavourings, further contribute to the intricate interplay influencing protein stability and structural transformations during processing (Marangoni, Barbut, McGauley, Marcone, & Narine, 2000). Hence, understanding the impact of shear flow on protein stability and developing reliable methods for testing and quantifying this influence is imperative. Such insights enable the informed design and selection of manufacturing processes, conditions, and formulations, ensuring optimal yields and stability in the production of protein-based products. The following sections explore the complexities of shear forces and how they influence the proteins within plant and dairy-based beverages, providing insights into the alterations in their structure and the consequential impact on physicochemical attributes.

2.8.1 Shear mechanisms

The flow of fluids is mainly categorized into extensional and shear flow (Bekard, Asimakis, Bertolini, & Dunstan, 2011; Perkins, Smith, & Chu, 1997). Homogeneous extensional flow exhibits a linear velocity gradient, $V_y = \dot{\gamma}y$, where V_y denotes the linear velocity, $\dot{\gamma}$ represents the velocity gradient, and y signifies the distance between the polymer centre and the flow axis. In contrast, simple shear flow involves a velocity gradient perpendicular to the flow field, combining rotational flow with vorticity (ω) and elongational flow with strain rate $\dot{\gamma} = \partial x_y/\partial y$. While rotational flow preserves protein structural integrity, elongational flow subjects proteins to stretching and compression events. Random exposure during these events leads to temporal fluctuations in molecular structures, including periodic elongation, relaxation, and tumbling. Stretching events expose structural elements to hydrodynamic shear stress, potentially causing unfolding. Increased shear rate intensifies molecular extension due to stronger hydrodynamic drag

(Bekard, Asimakis, et al., 2011; Bekard, Barnham, White, & Dunstan, 2011; Bekard & Dunstan, 2009). The exposure of hydrophobic sites in unfolded monomers initiates a reversible process of dimerization or oligomerization. When activators, including interfacial surfaces or impurities like other proteins are present, these activated oligomers form robust hydrophobic interactions, fulfill hydrogen bonding needs (such as interprotein β-sheets), overcome energy barriers, and give rise to irreversible yet soluble aggregates. Due to the complexity of these interactions, growth of these aggregates occurs through diverse mechanisms (Roberts, 2014). This dynamic interplay underscores the complex nature of molecular structures under shear flow conditions (Bekard, Barnham, et al., 2011).

The aggregation dynamics of proteins involves reversible and irreversible processes influenced by the collision frequency of unfolded monomers (Taboada-Serrano, Chin, Yiacoumi, & Tsouris, 2005). This phenomenon is characterized by three primary mechanisms: Brownian motion (perikinetic aggregation), orthokinetic aggregation, and differential settling (Meyer & Deglon, 2011). Smoluchowski's two-stage aggregation model describes particle kinetics in a flow, proposing equations for perikinetic (J_1) and orthokinetic (J_2) aggregation (Smoluchowski, 1918):

$$J_1 = \frac{4.k_B.T.N^2}{3.\eta} \tag{2}$$

$$J_2 = \frac{32}{3}.\alpha.N.d^3.\dot{\gamma} \tag{3}$$

The likelihood of irreversible interactions between particles depends on factors such as particle number (N), size, shear rate ($\dot{\gamma}$), and capture efficiency (α). Intermolecular bonds form when the collision frequency and energy are sufficiently high. Higher shear rates result in shorter contact times, limiting bond creation and favouring the formation of smaller particles (Zumaeta, Byrne, & Fitzpatrick, 2006). Flow-induced stresses play a significant role in aggregation kinetics but can also induce disruptive forces, especially impacting larger aggregates with potential weak spots. The equilibrium between growth and shear-controlled breakage determines the net growth rate and size of protein aggregates. Aggregation rates involve a two-stage process: diffusion-controlled

(perikinetic) followed by hydrodynamic-shear-controlled (orthokinetic) growth under hydrodynamic shear conditions (Taylor & Fryer, 1994).

Experimental flow techniques are typically divided into two primary categories based on flow type: capillary and rotational instruments. Common devices for analysing shear in rotational flow include cone and plate, parallel plate, and concentric cylinder viscometers. For this particular study, a concentric cylinder viscometer equipped with a pressure cell was employed to mitigate volatilization at elevated temperatures (Mediwaththe, 2017). In capillary systems, the flow exhibits a Poiseuille profile, with fluid being pushed through a tube of specific dimensions by creating a pressure differential from the entry to the exit point. This results in a non-uniform shear flow, featuring the highest shear rates at the fluid vessel's boundary and the lowest at the centre which gradually diminish inwards. The primary benefit of using rotational devices over capillary systems is their ability to maintain consistent shear rates across the solution, facilitating the analysis of time-dependent behaviours over prolonged periods.

2.8.2 Shear effects on milk proteins

During the processing of dairy fluids, various procedural steps, including pumping, stirring, mixing, UF, and homogenization expose the fluids to hydrodynamic shear stress. This type of stress on the proteins presents in these fluids, resulting in the destabilization of their native structures, ultimately leading to denaturation and aggregation (Bekard, Asimakis, et al., 2011; Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011). Shear rates encountered in dairy processing range from 10^1-10^4 s^{-1} in processes like mixing, stirring, pipe flow, and spray drying (Barnes, 1989; Šesták, Žitný, & Houška, 1983; Steffe, 1996).

2.8.2.1 Whey proteins

In their native state, whey proteins (WP) exhibit high solubility attributed to a significant presence of hydrophilic residues on the surface. However, exposure to denaturing agents including heat, pressure, and/or urea induces unfolding of these globular proteins, leading to subsequent aggregation. The rates and pathways of these physicochemical reactions are influenced by intrinsic protein characteristics and extrinsic environmental factors, including protein concentration, pH, temperature, ionic strength, and solvent conditions (Marangoni et al., 2000). The application of mechanical forces to proteins for

denaturation necessitates the use of exceptionally high shear rates (Thomas & Geer, 2011). The breakdown of aggregates under heightened shear stress can be attributed to various mechanisms. These include deformation and fragmentation caused by pressure fluctuations in fluid flows, erosion involving the abrasion of primary aggregates from particle surfaces, and the fragmentation of aggregates into larger fragments or as a consequence of these combined effects (Steventon, Donald, & Gladden, 2005; Taylor & Fryer, 1994).

Furthermore, the enforced rate of collision during shearing induces increased aggregation through distinct mechanisms. Initially driven by Brownian or thermal motion, the growth of aggregates is independent of shear stress whereby the motion triggers particle collisions, leading to the formation of primary aggregates. Once these primary aggregates attain a sufficient size, typically 0.25-1 μm, their motion becomes influenced by fluid flow, transitioning to hydrodynamically shear-controlled conditions. This shift increases the number of collisions between primary aggregates, elevating the probability of particle interaction and intensifying the overall aggregation process (Ker & Toledo, 1992; Simmons, Jayaraman, & Fryer, 2007; Taylor & Fryer, 1994).

2.8.2.2 Caseins

Caseins, integral phosphoproteins in milk, exhibit thermal stability and undergo precipitation at an isoelectric pH of 4.6 (Schmidt, 1982). The individual casein molecules are characterized by disorder and high flexibility, primarily attributed to their elevated proline content, which hinders the formation of a well-organized secondary structures (Holt et al., 2013). In milk, caseins exist in the form of micelles which are spherical and polydisperse particles with an average diameter of ~200 nm (Beliciu & Moraru, 2009; De Kruif, 1998). These micelles, occupying about two-thirds of the volume in solution, derive their structure from hydration of the porous matrix. This structural aspect contributes significantly to the generally heightened viscosity observed in casein suspensions (Dalgleish & Corredig, 2012).

The influence of shear flow extends to the structural integrity of casein micelles, potentially inducing transformations into more elongated shapes and displaying complex behaviours. Notably, at elevated concentrations, casein micelles exhibit characteristics resembling soft spheres, deforming and aligning along the direction of high shear rates

(Olivares, Berli, & Zorrilla, 2013). Additionally, the shear forces involved in various processes may impact the outer hydration layer of proteins, disrupting the stabilizing energy derived from preferential hydration. This disturbance can expose inner, non-polar residues, encouraging aggregation under increasing shear conditions (Stephen et al., 2008). The hydrophilic glycomacropeptide of κ-CN forms a hydration layer projecting into the aqueous phase of milk, contributing a negative charge that repels individual micelles and maintains their solubility in colloidal form without precipitation (Spreer & Mixa, 1998). The effect of shear on these hydration layers may lead to alterations in the overall charge distribution within the casein micelles, potentially resulting in flocculation which is a time-dependent process influenced by the number and efficiency of molecular collisions.

As discussed above, the detailed characteristics of both whey proteins and caseins under the influence of shear, will be further explored in relation to their behaviour under the influence of heat, pH, and protein or solids concentration, drawing from the most relevant and recent research conducted in this area.

2.8.3 Influence of heating

The current understanding suggests that shear-induced conformational changes initiate a range of weak interactions among constituent proteins, influencing their strength depending on the environment. Predominantly weak interactions involve Van-der Waals forces, molecular and steric repulsions, depletion interactions, hydrophobic interactions, and hydrogen bonds. However, for newly shear-formed structures to achieve full stabilization, strong covalent bonding (e.g. S-S bonds) is often required, and this is commonly achieved through the application of heat (Walstra et al., 2006). Upon heating up to approximately 65 °C, whey proteins undergo denaturation with β-LG unfolding reversibly which initiates aggregation. Around this temperature, a minor shift from α-helix to β-sheet structures occurs, enabling S-S interactions within the hydrophobic zone and inducing aggregation (Creamer et al., 2004). The free -SH group on Cys119 or Cys121 initiates SH-SS interchange reactions, affecting exposed SS bonds on other protein molecules by making them reactive and involving them in covalent interactions (Kinsella & Morr, 1984). Shear alone at typical food processing levels is insufficient to induce significant conformational changes in the secondary structure of WPs. Extremely high shear rates and/or turbulence are required for irreversible protein unfolding and

aggregation (Havea, Grant, Hii & Wiles, 2012). Controlling the rate and extent of WP denaturation, particularly of β-LG, is crucial for nucleation initiation and particle growth which can be further regulated by applying shear.

Several studies highlight the impact of simultaneous heating and shearing on WP aggregation complexity. Simmons et al. (2007) examined the effects of temperature (ranging from 70 to 90 °C) and shear rate (ranging from 111 s^{-1} to 625 s^{-1}) on the growth rate and size of aggregates in WPC solutions (WPC35) in relation to milk fouling. This study utilized a Couette apparatus and the variables studied included the addition of mineral calcium and phosphorus. At temperatures below 75 °C, the formed aggregates were observed to be small and weakly bonded. In contrast, higher temperatures, accompanied by increased shear rates, led to the formation of denser and more rigid aggregates. This distinction was attributed to weak Van der Waals bonding at lower temperatures and the development of stronger covalent disulfide bonds at elevated temperatures. The growth of aggregates was identified as a result of both protein denaturation and aggregation. Further temperature step-change experiments indicated that denaturation was highly dependent on temperature, while aggregation correlated with the applied shear field and particle strength. In addition, increasing the shear rate enhanced both denaturation and particle growth rates due to an augmented degree of particle collisions. However, the final particle size exhibited a complex behaviour with an increase in shear rate. The introduction of minerals into the WPC solution led to the formation of significantly smaller aggregates and increased deposition onto the surface of the Couette apparatus. This phenomenon was attributed to interactions between calcium and β-LG occurring both in the bulk solution and at the heated surface.

The shear-induced aggregation under the influence of heating was further studied by Erabit, Flick, & Alvarez (2014) who employed an innovative laboratory-scale apparatus to explore the impact of processing and compositional variables on the aggregation of β-LG. The experimental processing conditions included high protein concentrations (2-10% w/w), rapid heating (1 °C/s) and cooling (2 °C/s) rates, concentrated ionic environments (2.2-11 mM of $CaCl_2$), and shear rates (0-400 s^{-1}). In addition, variable holding times (0-240 s) and temperatures (67-95 °C) were systematically tested. The application of shear during heat treatment demonstrated a reduction in the concentration of small aggregates (1-10 μm) and an increase in the concentration of larger aggregates (20-200 μm).

A comprehensive study on shear induced behaviour of native milk proteins in raw milk when subjected to a range of temperatures and shear rates was reported by Mediwaththe, Bogahawaththa, Grewal, Chandrapala, & Vasiljevic (2018) and Mediwaththe, Chandrapala, & Vasiljevic (2018). Within these studies, the effect of shear under a range of temperatures (20-140 °C) and shear rates (100-1500 s^{-1}) were investigated. At the lower temperature of 20 °C, an increase in shear was observed to induce reversible changes in the protein structure. As the temperature was moderately increased to 72 °C, the proteins exhibited reversible structural alterations at a shear rate of 500 s^{-1}, which then transitioned to fragmentation and reformation processes at higher shear rates suggesting a dynamic response to shear stress. Progressing to higher temperatures, the effects of shear became more pronounced and irreversible. At temperatures reaching 120 and 140 °C, the shear force was found to predominantly cause the fragmentation of proteins. Notably, at the peak temperature of 140 °C, the shear further augmented the aggregation of proteins, predominantly through hydrophobic interactions, and to a lesser extent, thiol/disulfide bonding.

2.8.4 Influence of pH

The impact of altering the environmental pH on the characteristics of casein micelles within milk protein concentrate has been investigated by examining the influence of these conditions on the formation of differently sized particles with distinct physical attributes (Ranadheera et al., 2019). The effects of shear forces (100 and 1000 s^{-1}) were examined across a pH range from 2.0 to 7.5 and it was reported that at neutral pH levels of 6.7 and at 7.5, the particle size of casein micelles remains unchanged despite shearing. However, at pH levels lower than 4.6, the application of high shear resulted in a significant reduction in the size of casein aggregates which was particularly apparent at pH 2.0. The composition of the soluble phase also varied with pH, with an increase in β-casein at pH 4.6 and α_S-caseins at pH 2.0. The most substantial separation of caseins from the micelles was observed at the very acidic condition of pH 2.0, leading to the formation of small, soluble aggregates primarily composed of α_S- and β-caseins. Additionally, the structure of these aggregates was influenced by the level of shear applied, suggesting that the forces were inducing both the grouping together and breaking apart of particles.

Spiegel & Huss (2002) studied the combined effects of pH and calcium content on the denaturation kinetics and aggregation behaviour of whey proteins using a scraped surface

heat exchanger. They observed that the rate of β-LG denaturation at 80 °C was significantly slowed as the pH decreased from 6.7 to 4.5. The study also revealed that under shear conditions within a pH range of 4 to 5.5, the resulting aggregates were consistently small with a size <5 μm, regardless of lactose content or the temperature of heating. This phenomenon was attributed to the diminished reactivity of the thiol groups and the reduced net charge of the proteins at these specific pH levels. Additionally, when the calcium concentration was lowered, the combined heat and shear treatment led to the formation of a gritty texture characterized by large, rubbery particles. These particles were identified as fragments of a finely-stranded gel rather than primary whey protein aggregates, indicating complex interactions between the protein structure, calcium content, and processing conditions.

2.8.5 Influence of protein/solids concentration

Wolz, Mersch, & Kulozik (2016), investigated the impact of shear rate, heating time, and protein concentration on the particle characteristics of whey protein suspensions using a rotational rheometer at 80 °C. Elevating the protein concentration from 5 to 30% w/w resulted in the formation of smaller, more compact, and stable aggregates. This outcome can be attributed to the increased viscosity and higher shear stress in the system. The morphology of the aggregates transformed from a long, crystalline-like structures to spherical shapes. Moreover, the impact of shear rate was found to be dependent on the protein concentration. In a 5% protein suspension, the aggregate size initially increased with rising shear rates due primarily to an increased number of collisions. Subsequently, the aggregate size decreased indicating a limitation in particle growth under high shear. Conversely, at higher protein concentrations, the aggregate size decreased with shear rate signifying the influence of increasing shear stress. Therefore, the size of whey protein aggregates can be effectively controlled by adjusting the applied shear rate during processing.

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CHAPTER 3. Effect of Protein Content on Heat Stability of Reconstituted Milk Protein Concentrate under Controlled Shearing

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Published in *Foods* 2024, 13, 263

https://doi.org/10.3390/foods13020263

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Title of Paper/Journal/Book: Effect of Protein Content on Heat Stability of Reconstituted Milk Protein Concentrate under Controlled Shearing

Surname: Mediwaththe | First name: Anushka

Institute: Institute for Sustainable Industries and Liveab | Candidate's Contribution (%): 80

Status:

Accepted and in press: ☐ Date:

Published: ☑ Date: 14/01

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Todor Vasiljevic	10	Designing experiments, manuscript editing, revision and journal submission		14/02/24

Updated: September 2019

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Effect of Protein Content on Heat Stability of Reconstituted Milk Protein Concentrate under Controlled Shearing

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Abstract: Milk protein concentrates (MPCs) possess significant potential for diverse applications in the food industry. However, their heat stability may be a limitation to achieving optimal functional performance. Shearing, an inherent process in food manufacturing, can also influence the functionality of proteins. The aim of this research was to examine the heat stability of reconstituted MPCs prepared at two protein concentrations (4% and 8% *w*/*w* protein) when subjected to varying levels of shearing (100, 1000, or 1500 s^{-1}) during heating at 90 °C for 5 min or 121 °C for 2.6 min. While the impact of shear was relatively minor at 4% protein, it was more pronounced in 8% protein MPC suspensions, leading to a considerable decline in heat stability. An increase in protein concentration to 8% amplified protein interactions, intensified by shearing. This, in turn, resulted in comparatively higher aggregation at elevated temperatures and subsequently reduced the heat stability of the reconstituted MPCs.

Keywords: milk protein concentrate; protein concentration; heat stability; shear; aggregation

Citation: Mediwaththe, A.; Huppertz, T.; Chandrapala, J.; Vasiljevic, T. Effect of Protein Content on Heat Stability of Reconstituted Milk Protein Concentrate under Controlled Shearing. *Foods* **2024**, *13*, 263. https://doi.org/10.3390/foods13020263

Academic Editor: Ivano De Noni

Received: 30 December 2023
Revised: 11 January 2024
Accepted: 13 January 2024
Published: 14 January 2024

1. Introduction

Milk protein concentrates (MPCs) are milk protein ingredients that contain whey proteins and caseins in their original proportions as found in milk [1–3]. They are highly valued for their high protein content, milky flavor, and ability to add opacity to beverages. MPCs have a diverse range of applications, including inclusion in recipes for production of high-protein dairy beverages, as well as cheese, yogurt, ice cream, infant formula, and health-related products [4–9]. In these applications, MPC powders are reconstituted and subjected to heat treatments, such as pasteurization, ultra-high-temperature processing, or retort sterilization, wherein their heat stability plays a crucial role [10–12]. Heat stability is a key factor in achieving optimal functional performance in food systems, influencing properties such as solubility, emulsification, foaming, viscosity, water binding, gelling, freeze–thaw ability, and acid stability, which are strongly interrelated [13–16]. It is essential to evaluate the heat stability of reconstituted MPC powders to ensure consistent processing outcomes and maintain product quality by preventing adverse effects caused by heat-induced destabilization.

Factors such as temperature and duration of heating, as well as exposure to mechanical forces during, e.g., stirring, pumping, and homogenization, along with compositional factors such as protein concentration and presence of various other additives such as sugars or salts, can contribute to heat-induced destabilization [17–20]. Under such conditions, proteins in MPCs may undergo substantial chemical and physical changes leading to

aggregation, precipitation, sedimentation, or gelation, which can have detrimental effects on the quality of the final products.

Compositional factors play a significant role in heat-induced destabilization of milk and other milk protein-based systems [21]. It is widely recognized that the concentration of proteins affects the heat stability of milk protein systems. Concentrated milk typically exhibits lower heat stability compared to unconcentrated milk [22]. Similar observations have been made for MPCs, where greater protein concentration led to lower heat stability. This has been attributed to elevated levels of ionic calcium and increased viscosity [4,23,24]. Furthermore, the heat-induced formation of complexes between β-lactoglobulin (β-LG) and κ-casein (κ-CN) in the serum and colloidal phases of milk has been linked to heat stability issues [25,26].

Mechanical forces, such as shearing during the processing of products that contain MPCs, can also have a significant influence on the interactions between caseins and whey proteins. Shearing exposes protein structural elements to hydrodynamic shear stress, which can overcome stabilizing cohesive forces, such as intramolecular hydrogen bonds, resulting in protein unfolding [27–30]. Previous studies have indicated that shear has a substantial impact on the behavior of milk proteins across a wide range of temperatures [31].

Considering these findings, this study hypothesized that the heat stability of MPCs is profoundly influenced by the interplay of protein concentration, temperature, and shear. This hypothesis stemmed from the understanding that both heat and mechanical forces can cause substantial conformational changes in milk proteins, which in turn may affect the quality of the end product. Furthermore, there is a lack of research specifically examining the combined effects of temperature and shear on the heat stability of varying protein concentrations in MPCs. Therefore, the objective of this study was to investigate the influence of controlled shearing at different intensities (100, 1000, or 1500 s^{-1}) and temperatures (121 °C for 2.6 min or 90 °C for 5 min) at two different protein concentrations (4% and 8%) of MPCs commonly used in the production of dairy beverages. By examining these parameters, the study aimed to provide insights into the impact of shear and temperature on the heat stability of MPCs with varying protein concentrations.

2. Materials and Methods

2.1. Materials

The MPC was acquired from Fonterra Co-operative (Palmerston North, New Zealand) and stored at −20 °C in airtight plastic containers. MPC powder contained 81.0% total protein, 1.6% fat, 5.5% carbohydrate, and 7.2% ash, according to the manufacturer's declaration. All chemicals utilized in the analytical processes were sourced from Sigma-Aldrich Pty Ltd. (Castle Hill, NSW, Australia). Milli-Q water was used for all the experiments (Merck Millipore, Bayswater, VIC, Australia).

2.2. Preparation and Treatment of Samples

MPC suspensions with protein concentrations of 4% or 8% (*w*/*w*) were formulated by dissolving MPC powder in Milli-Q water. Each suspension was continuously stirred for 1 h at 50 °C for complete dispersion of the powder and continuously stirred at 4 °C overnight for complete hydration. Prior to commencing the experiments, the following day, the samples were allowed to equilibrate at a temperature of 25 °C for a duration of one hour [32]. Prepared MPC suspensions were heated at 121 °C for 2.6 min or 90 °C for 5 min at a constant shear rate of 0, 100, 1000, or 1500 s^{-1} in a pressure cell (CC25/PR-150) of a Physica MCR 301 series rheometer (Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with a constant pressure of 250 kPa using the method previously described [29]. Samples were heated at a rate of 5 °C min^{-1} to the required temperature and held there for the required time and cooled at a rate of 5 °C min^{-1}.

2.3. Particle Size and Zeta Potential Measurements

Following each treatment, particle size and zeta potential measurements were performed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) as described previously [32].

2.4. Fourier Transform Infrared (FTIR) Analysis

The changes in protein secondary structural features were assessed by FTIR spectrometer (Frontier, PerkinElmer, Waltham, MA, USA) analysis as described previously [32].

2.5. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS–PAGE)

After the treatments, a portion of all the treated and control samples was ultracentrifuged at 100,000× *g* for 1 h at 20 °C (Beckman Optima L-70 Ultracentrifuge, Indianapolis, IN, USA) to acquire the supernatant of the suspensions. Both non-reducing and reducing SDS–polyacrylamide gel electrophoresis (SDS–PAGE) were performed as described previously [32] for both bulk and supernatant MPC suspensions and stained with Coomassie Brilliant Blue (Sigma-Aldrich Pty Ltd., Castle Hill, NSW, Australia). The intensity of the treated reducing gel proteins in the supernatants was quantified as a percentage in relation to their corresponding protein content in the control bulk [32].

2.6. Determination of Protein Solubility and Heat Stability

The protein solubility at both concentrations was determined using the Kjeldahl method. After preparation of the suspensions as described in Section 2.2, these were centrifuged at 12,000× *g* for 20 min at 20 °C (model J2HS, Beckman, Fullerton, CA, USA) and supernatants were filtered through a 0.45 µm filter, following the procedure described previously [33]. A conversion factor of 6.38 for nitrogen was applied. Solubility was expressed as the protein content in the supernatant, relative to the total protein content in the original suspension, and expressed as a percentage [34,35].

The heat stability of each sample was evaluated by determining the protein content of the supernatant of samples before and after heat treatment. For this purpose, a portion of the sample was centrifuged at 12,000× *g* at 20 °C for 20 min (Model J2HS; Beckman, Fullerton, CA, USA) [34,35]. The protein content in the supernatants was determined using the Kjeldahl method, with a nitrogen conversion factor of 6.38. The heat stability of each suspension was then expressed based on these measurements as below:

% Heat Stability = (Protein content of the supernatant of heated MPC suspension)/(Protein content of the supernatant of unheated MPC suspension) × 100%

2.7. Statistical Analysis

The experimental design was replicated at least three times with subsequent subsampling. Statistical analysis was carried out with the use of IBM SPSS statistics software version 28.0.1.0 (IBM Corp., Armonk, NY, USA) using a multivariate general linear model (GLM) protocol as a randomized, blocked, split plot in time with protein concentration as the main factor and temperature/time combination and shearing as subplots. The replications served as a block. The threshold for significance was established at $p \leq 0.05$. The post hoc analysis for comparing multiple means was conducted using Tukey's Studentized range test (HSD). All the results were reported as mean ± standard error. Principal component analysis (PCA) was conducted for FTIR analysis using PCA for spectroscopy app, Origin Pro 2018 (v.95E) in the broad Amide I region (1700–1600 cm^{-1}) and in the region of 1200–900 cm^{-1} and score plots were obtained to group spectra for comparison purpose.

3. Results

3.1. Solubility and Heat Stability of Milk Protein Concentrations Subjected to Different Treatments

The solubility of MPC suspensions was comparable at both protein concentrations (Table 1). Heating both 4% and 8% protein MPC suspensions up to 121 °C resulted in a decline in heat stability, with both exhibiting similar levels at ~75% (Table 1).

Table 1. Particle size, zeta potential, solubility, and heat stability of 4% and 8% protein MPC suspensions subjected to shearing at 90 °C for 5 min or 121 °C for 2.6 min.

Protein (%)	Shear Rate (s^{-1})	Average Particle Size (nm)			Zeta Potential (mV)			Solubility %	Heat Stability %	
		25 °C	90 °C	121 °C	25 °C	90 °C	121 °C		90 °C	121 °C
4	0	190 ± 2^{Ba}	174 ± 1^{Bb}	177 ± 1^{Ab}	-21.1 ± 0.1^{Ba}	-22.7 ± 0.6^{Aa}	-21.7 ± 0.5^{Ca}	98.7 ± 0.1^{A}	85.5 ± 0.1^{Aa}	75.0 ± 0.9^{ABb}
	100		177 ± 1^{ABa}	176 ± 2^{Aa}		-22.0 ± 0.4^{Aa}	-22.7 ± 0.5^{Ca}		80.1 ± 0.6^{BCa}	74.6 ± 1.0^{ABb}
	1000		177 ± 2^{ABa}	175 ± 2^{Aa}		-22.6 ± 0.5^{Ab}	-24.2 ± 0.1^{Ba}		81.6 ± 0.3^{BCa}	78.2 ± 0.9^{Aa}
	1500		178 ± 2^{ABa}	175 ± 1^{Aa}		-21.3 ± 0.8^{Ab}	-25.9 ± 0.2^{Aa}		83.1 ± 0.1^{ABa}	80.1 ± 3.1^{Aa}
8	0	195 ± 1^{Aa}	178 ± 2^{ABb}	176 ± 2^{Ab}	-23.7 ± 0.2^{Ab}	-22.6 ± 0.5^{Aa}	-22.7 ± 0.5^{Ca}	95.4 ± 0.3^{B}	79.0 ± 1.1^{Ca}	75.0 ± 0.8^{ABb}
	100		178 ± 2^{ABa}	175 ± 1^{Aa}		-21.7 ± 0.5^{Aa}	-22.1 ± 0.4^{Ca}		75.3 ± 0.3^{Da}	70.2 ± 0.6^{BCb}
	1000		177 ± 1^{ABa}	175 ± 1^{Aa}		-21.6 ± 0.6^{Aa}	-22.8 ± 0.6^{Ca}		70.3 ± 0.5^{Ea}	65.6 ± 0.4^{CDb}
	1500		179 ± 1^{Aa}	176 ± 2^{Aa}		-21.5 ± 0.6^{Aa}	-22.5 ± 0.5^{Ca}		65.4 ± 0.1^{Fa}	60.0 ± 0.4^{Db}

The values are presented as means of subsampling from three independent observations, ±standard error. Values denoted with different uppercase letters within columns and different lowercase letters within rows for the same parameter indicate significant differences ($p < 0.05$).

The combined application of heat and shear exerted a noticeable impact on the heat stability of MPC suspensions. In the case of 4% protein MPC suspensions, heat stability decreased when subjected to low shear at 100 s^{-1} and at 90 °C down to ~80%. However, the heat stability increased to 83.1% when the shear rate was further raised to 1500 s^{-1}. Heat stability appeared unaffected when shear was applied at 121 °C in 4% protein MPC suspensions (Table 1). The combination of heat and shear had a significant effect on the heat stability of 8% protein MPC suspensions, its reduction was apparent at both temperatures and clearly shear-dependent. The decline was more pronounced at 121 °C, where heat stability dropped to ~60% at a shear rate of 1500 s^{-1} in comparison to other conditions.

3.2. Average Particle Size and Zeta Potential Measurements of Milk Protein Concentrations Subjected to Different Treatments

The initial average particle size of 4% protein MPC suspensions was ~190 nm after hydration (Table 1). As the temperature increased to 90 °C, the average particle size decreased to ~174 nm and remained constant with further temperature elevation to 121 °C (Table 1). Similarly, the 8% protein MPC suspension initially had an average particle size of ~195 nm (Table 1). This size decreased to ~178 nm at 90 °C and showed no significant change with a further temperature increase to 121 °C (Table 1). The simultaneous application of heat and shear had no significant impact on the average particle size within both 4% and 8% protein concentrations at both 90 °C and 121 °C (Figure 1 and Table 1).

The zeta potential of the 4% protein MPC suspension was ~−21 mV, and heating did not induce significant changes (Table 1). However, for the 8% protein MPC suspension, heating had a notable effect, reducing the negative zeta potential from ~−23.7 mV at 20 °C to ~−22.7 mV at 121 °C (Table 1). Simultaneous application of heat and shear resulted in no substantial change in the zeta potential in 4% protein MPC suspensions at 90 °C. However, at 121 °C, the zeta potential became more negative at both 1000 s^{-1} and 1500 s^{-1} shear rates. At 1500 s^{-1} shear rate, the zeta potential increased to ~−26 mV from ~−22 mV at 121 °C with no shear. Conversely, in the 8% protein MPC suspensions, the zeta potential remained unchanged at both temperatures when shear was applied.

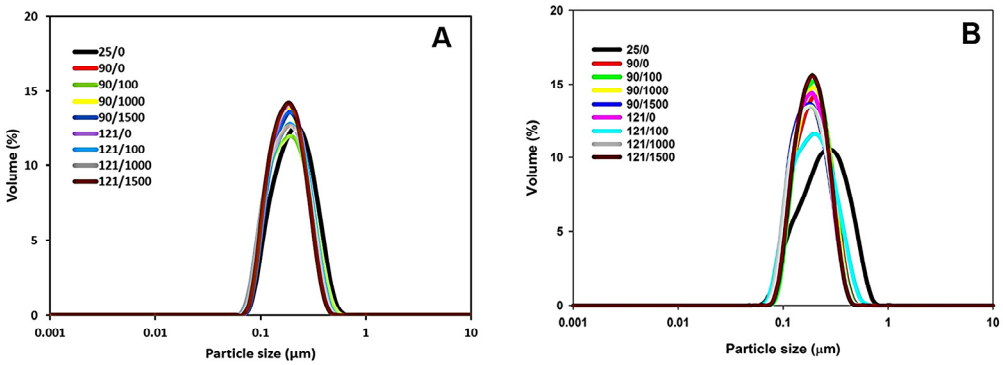

Figure 1. Particle size distribution of 4% protein MPC suspension subjected to heating at 90 or 121 °C and shear rate of 100, 1000, or 1500 s^{-1} (**A**) or 8% protein MPC suspension subjected to heating at 90 or 121 °C and shear rate of 100, 1000, or 1500 s^{-1} (**B**). The true controls were assessed prior to heating without shear (0 s^{-1}).

3.3. Secondary Structural Modifications of Milk Protein Concentrations Subjected to Different Treatments

In both 4% and 8% protein MPC suspensions, heating at 90 °C resulted in primarily slight variations in β-sheets and α-helices (Figure 2 and Table 2). The structural changes were more pronounced at 8% protein, with a reduction down to ~68% in α-helices and an increase up to ~67% in β-sheets at 121 °C, indicative of extensive aggregation. In comparison, at 4% protein, there was a reduction down to ~41% in α-helices and an increase up to ~62% in β-sheets at 121 °C. This was also accompanied by a substantial increase in

β-turns (~1667–1684 cm^{-1}) and a decrease in random structures (~1643–1646 cm^{-1}) at 8% concentration (Figure 2 and Table 2).

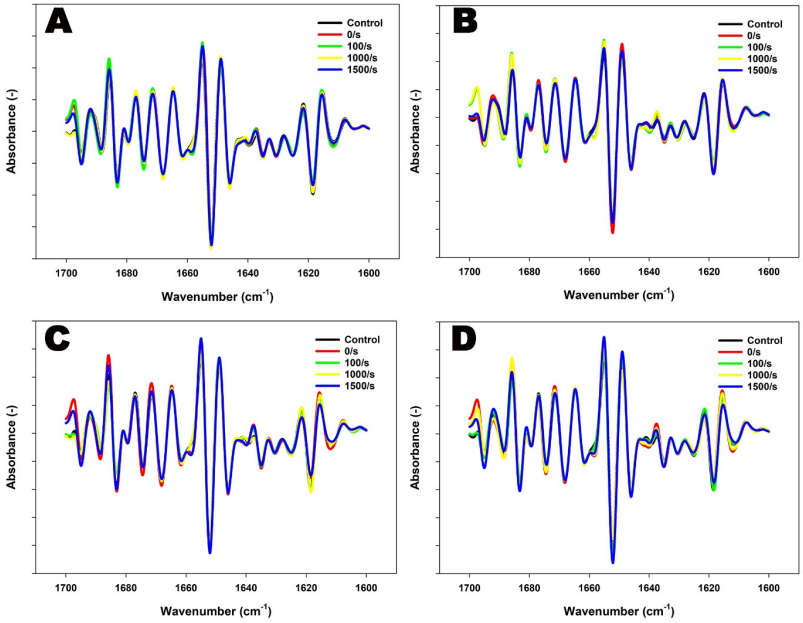

Figure 2. FTIR spectra (second derivative) for the Amide I region of 4% protein MPC suspensions sheared at 100, 1000, or 1500 s^{-1} during heating at 90 °C for 5 min (**A**) or 121 °C for 2.6 min (**B**), and 8% protein MPC suspensions sheared at 100, 1000, or 1500 s^{-1} during heating at 90 °C for 5 min (**C**) or 121 °C for 2.6 min (**D**). The true controls were assessed prior to heating without shear (0 s^{-1}).

Table 2. Total percentage areas of different secondary structures in the Amide I region of proteins in 4% and 8% protein MPC suspensions subjected to heat and shear treatments.

Protein (%)	Temp. (°C)	Shear Rate (s^{-1})	α-Helix (1646–1664 cm^{-1})	Total β-Sheet (1615–1637/1682–1700 cm^{-1})	Total β-Turns (1665–1681 cm^{-1})	Random (1638–1645 cm^{-1})
4	25	0	5.4 ± 0.2^{e}	43.1 ± 0.1^{n}	8.9 ± 0.1^{j}	42.6 ± 0.3^{b}
	90	0	4.1 ± 0.1^{h}	47.2 ± 0.3^{l}	7.4 ± 0.4^{k}	41.3 ± 0.1^{c}
		100	2.0 ± 0.3^{l}	52.7 ± 0.4^{i}	6.4 ± 0.1^{m}	38.9 ± 0.2^{d}
		1000	3.8 ± 0.5^{i}	46.8 ± 0.2^{m}	12.0 ± 0.1^{e}	37.4 ± 0.1^{e}
		1500	7.7 ± 0.1^{b}	48.8 ± 0.2^{k}	12.2 ± 0.1^{e}	31.3 ± 0.4^{f}
	121	0	3.2 ± 0.1^{j}	69.9 ± 0.3^{b}	15.8 ± 0.4^{b}	11.1 ± 0.3^{p}
		100	5.4 ± 0.4^{e}	67.4 ± 0.1^{d}	13.3 ± 0.2^{d}	13.9 ± 0.5^{n}
		1000	4.8 ± 0.1^{f}	70.9 ± 0.4^{a}	11.2 ± 0.7^{f}	13.1 ± 0.1^{o}
		1500	4.7 ± 0.2^{fg}	68.5 ± 0.1^{c}	10.2 ± 0.1^{h}	16.6 ± 0.7^{m}
8	25	0	8.0 ± 0.4^{b}	35.8 ± 0.1^{o}	6.8 ± 0.5^{l}	49.4 ± 0.1^{a}
	90	0	6.2 ± 0.1^{d}	50.4 ± 0.2^{j}	15.2 ± 0.1^{c}	28.2 ± 0.4^{g}
		100	10.0 ± 0.7^{a}	57.8 ± 0.2^{h}	10.4 ± 0.3^{h}	21.8 ± 0.3^{j}
		1000	7.2 ± 0.1^{c}	61.8 ± 0.2^{f}	10.0 ± 0.3^{i}	21.0 ± 0.1^{k}
		1500	7.1 ± 0.8^{c}	66.4 ± 0.5^{e}	7.1 ± 0.1^{kl}	19.4 ± 0.3^{l}
	121	0	2.6 ± 0.1^{k}	59.7 ± 0.2^{g}	17.9 ± 0.2^{a}	19.8 ± 0.2^{l}
		100	2.8 ± 0.3^{jk}	57.6 ± 0.2^{h}	16.0 ± 0.1^{b}	23.6 ± 0.1^{i}
		1000	4.4 ± 0.2^{fgh}	61.2 ± 0.1^{f}	10.8 ± 0.3^{g}	23.6 ± 0.3^{i}
		1500	4.3 ± 0.1^{gh}	67.2 ± 0.2^{d}	3.9 ± 0.1^{n}	24.6 ± 0.1^{h}

The data are represented as mean values ± standard error, derived from a minimum of three independent replications. Significant differences within a column are denoted by different lower-case superscript letters at $p < 0.05$.

The application of shear-induced protein secondary structural changes, with the extent of these changes dependent on the applied conditions as well as protein content within

suspensions (Figure 2 and Table 2). The combined application of heat and shear resulted in noticeable changes in the content of β-sheets in 4% protein suspensions. At 90 °C, intense peaks at ~1688 cm^{-1} were observed at a shear rate of 100 s^{-1}, demonstrating intermolecular and anti-parallel β-sheet-driven aggregation. These peaks were comparatively less pronounced at shear rates of 1000 s^{-1} and 1500 s^{-1}, suggesting a dominance of fragmentation of aggregates [36] (Figure 2 and Table 2). A further increase in temperature to 121 °C revealed the prevalence of shear-induced fragmentation. An increase in shear up to 1500 s^{-1} resulted in a ~50% increase in random structures, suggesting the dominant fragmentation of aggregates at 121 °C (Table 2).

On the other hand, the combined application of heat and shear to 8% protein MPC suspensions resulted in considerable β-sheet-driven aggregation at 90 °C, peaking at 1500 s^{-1}, resulting in a 32% increase in β-sheet content [36]. Furthermore, reduced peaks at ~1675 cm^{-1} and ~1624 cm^{-1} denote a reduced presence of β-turns and decreased intramolecular crosslinking consequently [37] (Figure 2 and Table 2). Further heating up to 121 °C in combination with shear also revealed the prevalence of shear-induced aggregation in 8% protein suspensions. An intense peak at ~1655 cm^{-1} at 1500 s^{-1} at 121 °C denotes possible shear-induced reformation of α-helical structures (Figure 2). Total β-sheets increased by ~13% at 1500 s^{-1}, suggesting further aggregation of proteins at 121 °C (Table 2).

These variations in the second derivative spectra of the Amide I region were further analyzed using principal component analysis (PCA). The PCA separated samples based on the shear rates at different concentrations and temperatures. However, no distinguishable separation of spectra was observed, suggesting subtle changes in secondary structural modifications that did not significantly contribute to the overall variance in the dataset (Supplementary Figure S1).

To explore the effects of treatments on the behavior of lactose and minerals, particularly phosphate under the given conditions, the FTIR spectral region of 1200–900 cm^{-1} was also subjected to analysis. In 4% protein suspensions, distinguishable peaks were observed at ~970 cm^{-1} and at ~990 cm^{-1} at both 1000 s^{-1} and 1500 s^{-1} and at 90 °C (Figure 3). When temperature was further increased to 121 °C, two prominent peaks at ~970 cm^{-1} and ~987 cm^{-1} were observed at 1500 s^{-1}.

Similar to 4% protein suspensions, prominent peaks were detected at both ~970 cm^{-1} and 990 cm^{-1} in 8% protein suspensions sheared at 1500 s^{-1} and 90 °C within the region of 1200–900 cm^{-1}. At 121 °C, a distinguishable peak ~990 cm^{-1} was observed at 1500 s^{-1} (Figure 3). These observations were further supported by PCA analysis performed in the region of 1200–900 cm^{-1}, which explained over 85% of the variance and captured most of the spectral changes.

3.4. Partitioning of Proteins in Milk Protein Concentrate Subjected to Different Treatments

Protein interactions were further analyzed using non-reducing and reducing SDS–PAGE. In both 4% and 8% protein, the presence of aggregates was observed in all treatments at both 90 °C and 121 °C. These aggregates, which were observed on top of non-reducing stacking gels, completely disappeared under reducing conditions, indicating that they were exclusively formed as a result of thiol/disulfide interactions (Figures 4 and 5). Heating both 4% and 8% protein MPC suspensions resulted in a gradual decrease in β-LG and α-LA concentrations, with no notable difference between the two concentrations (Table 3). Conversely, all caseins (α_s-CN, β-CN, and κ-CN) in the supernatant increased, and there was no significant difference observed between the two concentrations (Table 3).

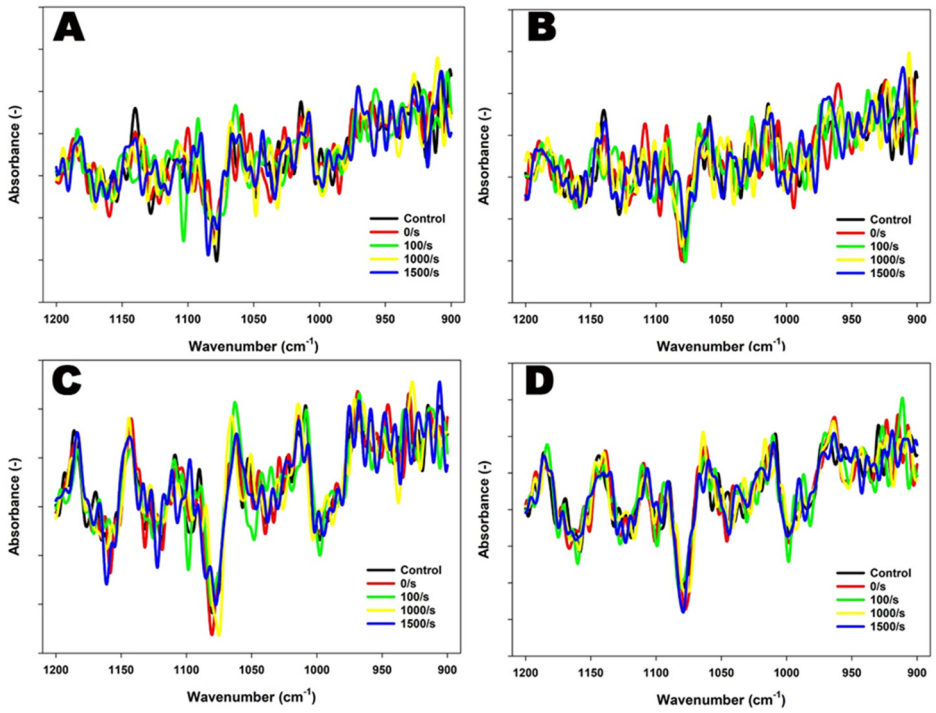

Figure 3. FTIR spectra of 4% protein MPC suspensions sheared at 100, 1000, or 1500 s^{-1} during heating at 90 °C for 5 min (**A**) or 121 °C for 2.6 min (**B**), and 8% protein MPC suspensions sheared at 100, 1000, or 1500 s^{-1} during heating at 90 °C for 5 min (**C**) or 121 °C for 2.6 min (**D**), obtained in the region between 1200 and 900 cm^{-1}. The true controls were assessed prior to heating without shear (0 s^{-1}).

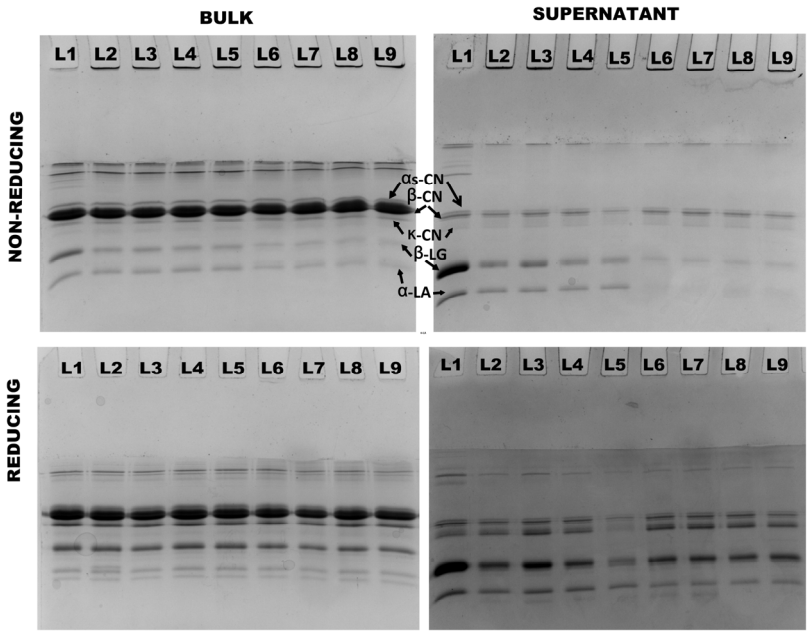

Figure 4. Non-reducing and reducing SDS–PAGE analysis of bulk and supernatant of 4% protein MPC suspensions (Lane sequence—(25-0)/90-0/90-100/90-1000/90-1500/121-0/-121-100/121-1000/121-1500 from left to right).

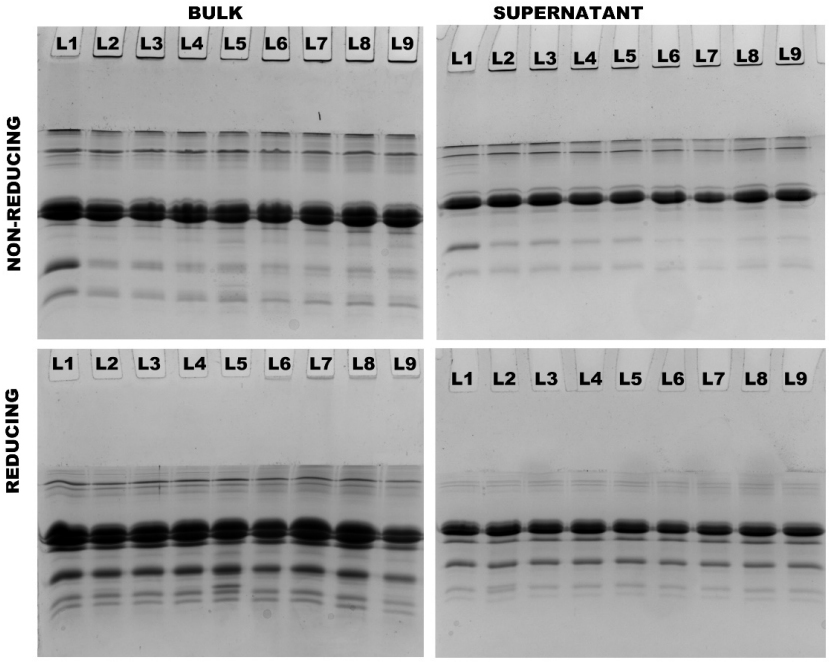

Figure 5. Non-reducing and reducing SDS–PAGE analysis of bulk and supernatant of 8% protein MPC suspensions (Lane sequence—(25-0)/90-0/90-100/90-1000/90-1500/121-0/-121-100/121-1000/121-1500 from left to right).

Table 3. Intensity of individual caseins, β-LG, and α-LA in supernatants of 4% and 8% protein MPC suspensions as a % of their intensity in the respective control bulk suspensions subjected to different treatments resolved under reducing electrophoretic conditions and quantified using a ChemiDoc imager.

Protein (%)	Temp. (°C)	Shear Rate (s^{-1})	α_s-CN	β-CN	κ-CN	β-LG	α-LA
4	25	0	7.5 ± 0.1^{h}	9.8 ± 0.4^{f}	28.7 ± 1.2^{i}	99.3 ± 0.6^{a}	99.7 ± 0.1^{a}
	90	0	8.1 ± 0.3^{f}	10.3 ± 0.1^{e}	31.3 ± 0.9^{h}	55.1 ± 0.8^{f}	63.8 ± 0.9^{f}
		100	8.8 ± 0.2^{d}	9.7 ± 0.7^{f}	38.2 ± 0.4^{e}	56.2 ± 0.3^{f}	64.2 ± 0.7^{f}
		1000	8.3 ± 0.1^{e}	9.2 ± 0.6^{g}	45.2 ± 0.7^{d}	67.8 ± 0.4^{d}	71.3 ± 0.4^{d}
		1500	7.8 ± 0.4^{g}	8.9 ± 0.2^{h}	48.5 ± 0.5^{c}	75.0 ± 0.2^{b}	78.3 ± 0.5^{c}
	121	0	10.4 ± 0.2^{b}	12.5 ± 0.1^{a}	35.3 ± 0.2^{f}	50.4 ± 0.9^{g}	59.3 ± 0.2^{h}
		100	11.3 ± 0.7^{a}	11.8 ± 0.9^{b}	37.8 ± 0.4^{e}	51.5 ± 1.1^{g}	61.5 ± 0.5^{g}
		1000	10.5 ± 0.9^{b}	10.9 ± 1.1^{c}	51.3 ± 0.5^{b}	70.3 ± 0.7^{b}	79.8 ± 0.2^{c}
		1500	9.8 ± 0.2^{c}	10.6 ± 0.5^{d}	58.2 ± 0.1^{a}	73.8 ± 0.5^{b}	81.3 ± 0.4^{b}
8	25	0	6.8 ± 0.2^{k}	8.1 ± 0.5^{i}	20.3 ± 0.5^{k}	98.7 ± 0.2^{a}	99.1 ± 0.6^{a}
	90	0	7.2 ± 0.1^{i}	9.3 ± 0.3^{g}	23.4 ± 0.1^{j}	58.3 ± 0.9^{e}	65.1 ± 0.5^{f}
		100	7.0 ± 0.5^{j}	7.3 ± 0.2^{j}	29.7 ± 0.9^{i}	57.5 ± 0.7^{ef}	63.8 ± 0.3^{f}
		1000	5.3 ± 0.3^{m}	6.5 ± 0.7^{l}	32.5 ± 0.7^{g}	59.1 ± 0.4^{e}	68.1 ± 0.4^{e}
		1500	4.9 ± 0.4^{n}	5.9 ± 0.2^{m}	33.8 ± 0.8^{g}	68.5 ± 0.5^{cd}	73.2 ± 0.9^{d}
	121	0	10.5 ± 0.8^{b}	10.3 ± 0.7^{e}	30.8 ± 0.5^{hi}	47.3 ± 0.3^{h}	58.5 ± 0.4^{h}
		100	9.8 ± 0.5^{c}	10.2 ± 0.2^{e}	31.3 ± 0.4^{h}	45.8 ± 0.2^{h}	56.3 ± 0.1^{i}
		1000	7.5 ± 0.3^{h}	6.8 ± 0.7^{k}	35.7 ± 0.2^{f}	52.3 ± 1.3^{g}	58.5 ± 1.3^{h}
		1500	6.1 ± 0.2^{l}	5.3 ± 0.3^{n}	37.2 ± 0.5^{e}	55.8 ± 0.9^{f}	61.8 ± 0.4^{g}

Values represent the means derived from a minimum of three independent replications, ±standard error. Significant differences within a column are denoted by different lower-case superscript letters at $p < 0.05$.

The application of heat and shear resulted in an increased concentration of β-LG, α-LA, and κ-CN in the supernatants of both 4% and 8% protein MPC suspensions heated at both 90 °C and 121 °C, compared to the suspensions subjected solely to heat treatment.

Notably, this increase was more pronounced in the 4% protein suspensions than in the 8% protein MPC suspensions (Table 3). Particularly, κ-CN exhibited a higher dissociation in 4% protein suspensions compared to 8% protein suspensions at the highest shear rate of 1500 s^{-1} at 121 °C.

In 4% protein suspensions, at 121 °C, increasing the shear from 0 s^{-1} to 1500 s^{-1} resulted in a ~23% increase in β-LG, a ~22% increase in α-LA, and a ~23% increase in κ-CN levels in the supernatant. On the other hand, in 8% protein suspensions at 121 °C, raising the shear from 0 s^{-1} to 1500 s^{-1} led to a ~9% increase in β-LG, a ~3% increase in α-LA, and a ~6% increase in κ-CN levels in the supernatant (Table 3).

4. Discussion

In both 4% and 8% protein MPC suspensions, micellar casein dissociation and the formation of smaller whey protein aggregates with an increase in temperature were evident, as observed by SDS–PAGE data showing elevated levels of soluble caseins and a decrease in whey proteins in the soluble phase (Table 3). However, the level of aggregation during heating did not appear to be influenced by the protein content at either temperature. The micellar casein dissociation and formation of smaller whey protein aggregates may have led to a reduction in average particle size at both concentrations during heating [37] (Table 1). However, this reduction in particle size with heating was not significantly affected by the heating temperature or the protein content within the suspension. Additionally, while the heat stability gradually decreased as the temperature increased at both 4% and 8% protein concentration, it was not influenced by the protein content, especially at 121 °C, where comparable levels were reached in both suspensions (Table 1). These observations were further supported by FTIR analysis, where no noticeable difference in the extent of heat-induced changes was observed in both concentrations that could be attributed to their protein content, as evidenced by PCA analysis. These findings align with previous studies by [38], where the rate constants for the thermal denaturation of β-LG were known to be independent of the initial protein concentration in skim milk systems. Notably, it was established that, at higher temperatures (>~90 °C), the rate constants remained independent of the whey protein concentration [38–40]. This implies that the heat stability of proteins was not markedly influenced by the initial protein concentration under the given conditions. The fact that the denaturation rate constant remained unaffected by the initial protein concentration may contribute to the observed uniformity in heat stability. However, it is important to note that the rate of denaturation, while a factor, may not be the primary influence on heat stability. Other factors or interactions could also be influential in determining the overall stability of the system subjected to heat treatment [23].

The combined application of heat and shear resulted in increased levels of β-LG, α-LA, and κ-CN in the soluble phase, indicating significant shear-induced fragmentation of aggregates in both suspensions (Table 3). Fluid dynamics play a crucial role in affecting aggregation kinetics and can introduce disruptive stresses that lead to the breakdown of agglomerates [41–45]. This shear-induced fragmentation occurs through controlled fragmentation of complexes due to pressure fluctuations in fluid flow, particle fragmentation from larger complexes, and abrasion of primary complexes from newly created particle surfaces [46,47]. In the case of 4% protein suspension, higher levels of β-LG, α-LA, and κ-CN were observed in the soluble phase, indicating a pronounced impact of shear on cohesive forces, including hydrophobic, van der Waals, or electrostatic interactions between particles (Table 3). The FTIR data in 4% protein MPC suspensions provided further evidence of shear-induced fragmentation, as shown by reduced peak intensities of intermolecular β-sheets and the presence of unordered structures, particularly at high shear rates of 1000 s^{-1} and 1500 s^{-1} (Figure 2 and Table 2). Furthermore, the dissociation of caseins was prominent in 4% protein MPC suspensions when subjected to shear, as supported by FTIR results, which showed two concurrent peaks at ~990 cm^{-1} and ~980 cm^{-1} at both temperatures (Figure 3). The appearance of the latter band may indicate the release of the MCP nanoclusters from the casein micelle, resulting in an increased negative charge of the casein molecules, partic-

ularly observed at 121 °C, where there was a substantial increase in negative zeta potential at both 1000 s^{-1} and 1500 s^{-1} shear rates compared to control (Table 1). The presence of the former band suggests the dissociation of the MCP into Ca^{2+} and HPO_4^{2-} upon release from the serine phosphate residues. The intensities of these two bands correlate with the micelle dissociation process. In addition, this was supported by SDS–PAGE analysis, revealing a prominent increase in κ-CN content in the soluble phase at the highest shear rate of 1500 s^{-1} at 121 °C in 4% protein suspensions, which could be attributed to shear-induced casein dissociation (Table 3). Shear flow exerts fluid drag, disrupting and destabilizing casein micelles, making them more susceptible to dissociation, as observed in previous studies [31,48]. This shear-induced micellar dissociation and fragmentation of aggregates were further supported by particle size data, demonstrating a reduction in particle size, especially at 121 °C, suggesting a more pronounced effect (Table 1). However, in 4% protein suspensions, heat stability decreased at 100 s^{-1} and 90 °C. A further increase in shear up to 1500 s^{-1} increased the heat stability. Nevertheless, heat stability was unaffected by increased shear at 121 °C. As observed, applied shear can disrupt protein components and modify their structure, depending on its magnitude, leading to alterations in the distribution of surface-active proteins within the concentrate [49,50] and contributing to variations in the heat stability of MPC suspensions.

When compared to 4% protein suspensions, shear-induced fragmentation was observed to be less pronounced in 8% protein MPC suspensions. This could be due to either resistance to applied shear forces dominating as cohesive stabilization or shear-induced aggregation becoming dominant up to a certain extent. The process of aggregation, breakup, and restructuring in a dispersion is intricately influenced by the balance between hydrodynamic forces and cohesive forces [51–54]. At high shear rates, hydrodynamic drag forces disrupt cohesive forces, such as intramolecular hydrogen bonds, leading to protein unfolding. Consequently, exposed reactive sites facilitate the formation of complexes between whey proteins and micellar caseins, resulting in further aggregation at elevated temperatures [55–57]. As the milk protein concentration increases, the likelihood of protein–protein interactions rises, leading to protein aggregation and the formation of larger complexes due to the influence of shear. The prominent aggregation with an increase in shear in 8% protein suspensions is evident from the FTIR data, which indicates an increase in intermolecular β-sheets as a result of β-sheet-driven aggregation, along with a decrease in random structures, particularly at 121 °C, compared to heated controls (Table 2) [58]. In addition, comparatively less release of β-LG, α-LA, and κ-CN into the soluble phase (Table 2), as well as no observable effect on particle size with an increase in shear compared to 4% protein suspensions (Table 1), specifically explains a certain level of aggregation in 8% protein MPC suspensions compared to 4% protein suspensions. Furthermore, the stabilizing effect against heat-induced changes may become less effective due to the effect of hydrodynamic shear, which improves particle collisions. The growth rate and size of protein aggregates are influenced by the balance between growth and controlled shear-induced breakage. Shear-induced growth (orthokinetic aggregation) in shear flow directly correlates with shear rate, particle quantity, and capture efficiency, with the latter linked to medium viscosity [59–62]. Owing to its higher total solid content and increased particle concentration, 8% protein MPC suspension exhibits elevated viscosity compared to the 4% protein MPC suspension. This higher viscosity further enhances the system's resistance to shear forces, subsequently modulating particle interactions. Consequently, the 8% protein MPC suspension demonstrates pronounced aggregation in contrast to the 4% protein MPC suspension, which experiences fragmentation. The larger complexes that are formed can disrupt the original structure, rendering the system more susceptible to coagulation, gelation, and other forms of destabilization when exposed to heat, ultimately resulting in reduced heat stability. Reduced heat stability was observed at both 90 °C and 121 °C, along with the increase in shear, resulting in the lowest levels at 1500 s^{-1} and at 121 °C (Table 1). Collectively, these multifaceted factors collaborate to govern the ultimate size, structural configuration, and stability of particles within the given system.

5. Conclusions

Shearing has the ability to modify the structure and physicochemical properties of milk proteins, which, in turn, can affect the mechanisms of protein interactions and aggregation during heating and impact heat stability. When comparing 8% protein MPC suspensions to 4% protein suspensions, it was observed that higher shear levels result in lower heat stability in 8% protein suspensions. The increase in protein concentration up to 8% enhances protein interactions, making proteins more prone to aggregate formation during high-temperature heating. At high shear rates, cohesive stabilizing forces, such as intramolecular hydrogen bonds that maintain the helical structure, are disrupted by hydrodynamic drag forces, leading to molecular unfolding. As a result, previously hidden reactive sites are exposed, promoting the formation of complexes between whey proteins and casein micelles. This, in turn, leads to further aggregation at high temperatures and ultimately reduces heat stability. Therefore, considering the given parameters, low protein content (4%) and low temperatures would be most suitable for providing optimal heat stability under high shear conditions in reconstituted milk products. In addition, it is known that commercial MPCs vary greatly in terms of their gross composition including protein and mineral content, which can also govern the course of denaturation and/or aggregation of proteins upon heating and shearing. For this reason, a wider range of commercial MPCs should be assessed in order to generalize the observations and conclusions of this study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods13020263/s1, Figure S1: Score plots of principal component analysis of FTIR spectra obtained within the Amide I region from 4% RMPC dispersions sheared at 100 s^{-1}, 1000 s^{-1}, or 1500 s^{-1} during heating at 90 °C for 5 min (A) or 121 °C for 2.6 min (B), and 8% MPC suspensions sheared at 100 s^{-1}, 1000 s^{-1}, or 1500 s^{-1} during heating at 90 °C for 5 min (C) or 121 °C for 2.6 min (D).

Author Contributions: Conceptualization, A.M.; Data curation, A.M.; Formal analysis, A.M.; Investigation, A.M.; Methodology, A.M., J.C. and T.V.; Project administration, T.V.; Resources, T.V.; Supervision, T.H., J.C. and T.V.; Validation, T.H. and T.V.; Writing—original draft, A.M.; Writing—review and editing, T.H., J.C. and T.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article or Supplementary Material.

Acknowledgments: The authors extend their appreciation to Victoria University for the financial and technical assistance provided.

Conflicts of Interest: Thom Huppertz is employed by the company FrieslandCampina. The research was conducted without any commercial and financial relationships with the company that could be construed as a potential conflict of interest.

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Supplementary Material

Effect of Protein Content on Heat Stability of Reconstituted Milk Protein Concentrate under Controlled Shearing

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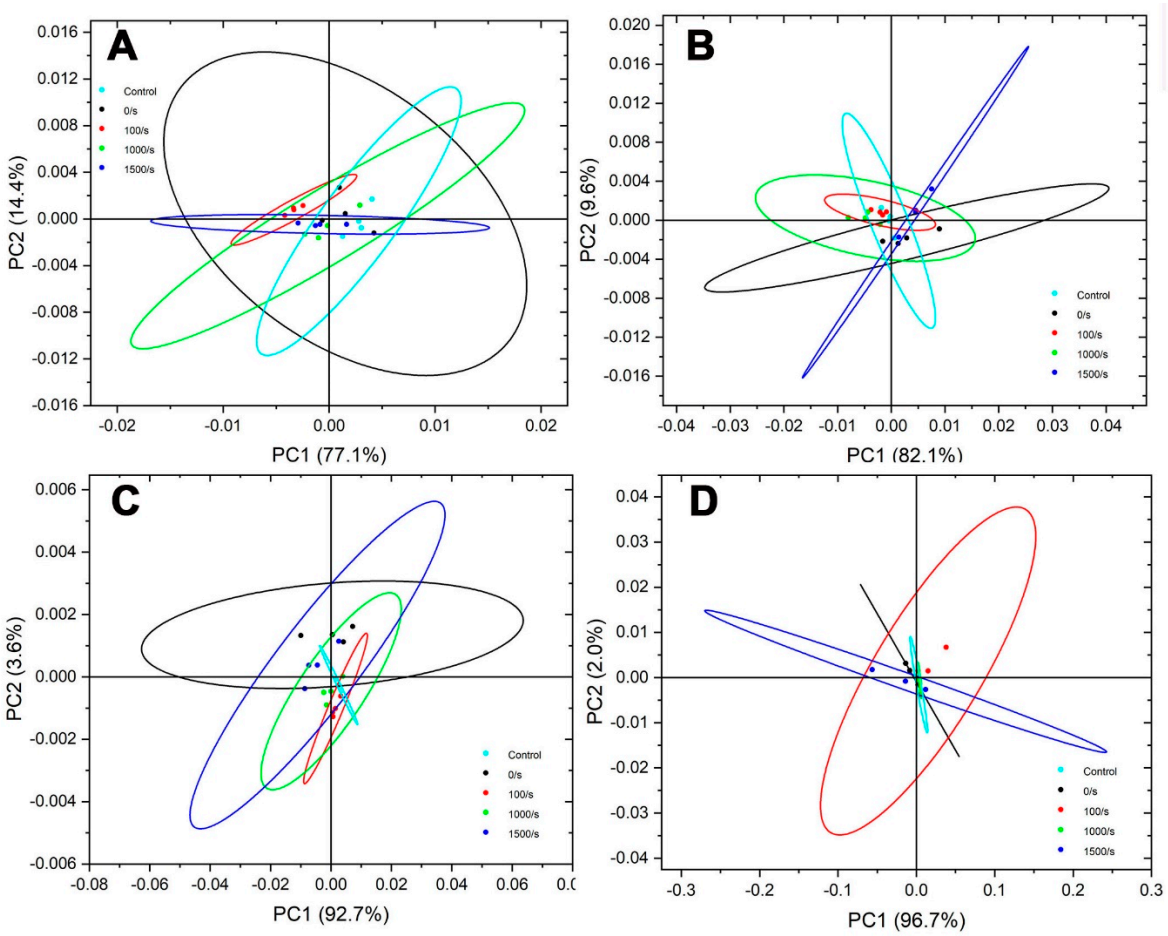
Content

Figure S1. Score plots of principal component analysis of FTIR spectra obtained within the Amide I region from 4% RMPC dispersions sheared at 100 s^{-1}, 1000 s^{-1} or 1500 s^{-1} during heating at 90 °C for 5min. (A) or 121°C for 2.6 min (B) and 8% MPC suspensions sheared at 100 s^{-1}, 1000 s^{-1} or 1500 s^{-1} during heating at 90 °C for 5min. (C) or 121°C for 2.6 min (D).



CHAPTER 4. Effect of pH and Shear on Heat-Induced Changes in Milk Protein Concentrate Suspensions

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Published in *Foods* 2024, 13, 1517

https://doi.org/10.3390/foods13101517

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Title of Paper/Journal/Book: Effect of pH and Shear on Heat-Induced Changes in Milk Protein Concentrate Suspensions

Surname: Mediwaththe First name: Anushka

Institute: Institute for Sustainable Industries and Liveab Candidate's Con 80

Status:

Accepted and in press: ☐ Date:

Published: ✔ Date: 13/05

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Anushka Mediwaththe	80	Experiment design and execution, data analysis, manuscript drafting and	[redacted]	16/05/24
Thom Huppertz	5	Designing experiments, manuscript editing and revision		17.05.2024
Jayani Chandrapala	5	Designing experiments, manuscript editing and revision		17.05.2024
Todor Vasiljevic	10	Designing experiments, manuscript editing,revision and journal submission		17.05.2024

Updated: September 2019

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Effect of pH and Shear on Heat-Induced Changes in Milk Protein Concentrate Suspensions

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Abstract: The effect of shear on heat-induced changes in milk protein concentrate suspensions was examined at different pH levels, revealing novel insights into micellar dissociation and protein aggregation dynamics. Milk protein concentrate suspensions, adjusted to pH of 6.1, 6.4, 6.8, or 7.5, underwent combined heat (90 °C for 5 min or 121 °C for 2.6 min) and shear (0, 100, or 1000 s^{-1}) treatment. The fragmentation of protein aggregates induced by shear was evident in the control MPC suspensions at pH 6.8, irrespective of the temperature. At pH 7.5, shear increased the heat-induced micellar dissociation. This effect was particularly pronounced at 121 °C and 1000 s^{-1}, resulting in reduced particle size and an elevated concentration of κ-casein (κ-CN) in the non-sedimentable phase. At pH 6.1 or 6.4, shear effects were dependent on sample pH, thereby modifying electrostatic interactions and the extent of whey protein association with the micelles. At pH 6.1, shear promoted heat-induced aggregation, evidenced by an increase in particle size and a significant decline in both whey proteins and caseins in the non-sedimentable phase. At pH 6.4, shear-induced fragmentation of aggregates was observed, prominently due to comparatively higher electrostatic repulsions and fewer protein interactions. The influence of shear on heat-induced changes was considerably impacted by initial pH.

Keywords: dairy; milk protein concentrate; shear effect; temperature; pH variation

Citation: Mediwaththe, A.; Huppertz, T.; Chandrapala, J.; Vasiljevic, T. Effect of pH and Shear on Heat-Induced Changes in Milk Protein Concentrate Suspensions. *Foods* **2024**, *13*, 1517. https://doi.org/10.3390/foods13101517

Academic Editor: Agostino Sevi

Received: 16 April 2024
Revised: 11 May 2024
Accepted: 11 May 2024
Published: 13 May 2024

1. Introduction

Milk protein concentrates (MPCs) are typically produced by the ultrafiltration of pasteurized skim milk, resulting in a concentrated mixture rich in casein and whey proteins, which is then typically dried to an MPC powder. During this process, the permeate stream, which contains lactose, water, and (soluble) milk salts, is removed [1]. While the protein-to-total-solids ratio is increased in the MPC, the casein-to-whey-protein ratio remains similar to that of skim milk [1,2]. These characteristics of MPC make it suitable for high-protein dairy beverages, which often undergo UHT (ultra-high temperature) and retort sterilization to enhance their shelf life [3,4].

The stability of milk proteins and their susceptibility to denaturation and aggregation during thermal processing depends on various factors, including heating temperature and time, mechanical forces like shear, pH, and the relative abundance of proteins and minerals [5,6]. Changes in pH can influence attractive and repulsive electrostatic interactions between protein molecules, thereby also impacting the structure of the casein micelles. The modification of milk pH prior to heat treatment affects the degree of association of denatured whey proteins with micelles [7]. After heat treatment of milk with a pH < 6.8, a substantial proportion of the whey protein is found attached to the casein micelles. After heating milk with a pH > 6.8, whey protein aggregates are largely found in

the serum, upon association with κ-CN and dissociation from the casein micelles. However, heat-induced dissociation of micellar κ-CN in MPC occurs to a lesser extent than in milk, and only at higher pH values; it has been observed that at a pH > 6.8, the destabilizing effects of increased calcium ion activity due to the higher protein content in MPC powders ranging from MPC35 to MPC90 are partially offset by a decrease in heat-induced κ-CN dissociation [8,9].

While pH adjustment seems an obvious strategy to manipulate the heat stability of MPC, combining it with the simultaneous application of shear may create certain properties that would modify their structural characteristics, eventually leading to more pronounced effects. High shear could lead to structural changes in the casein micelle, e.g., either elongation along with the fluid drag or swelling, which would potentially improve the association of micellar caseins with the outer environment [10]. The intricate interplay between pH and shear dynamics was especially evident at low pH; for example, at pH 4.6 and particularly at pH 2.0, a notable reduction in the size of casein aggregates was observed with high shearing [11]. In the serum, these caseins were present as relatively small, soluble aggregates, primarily composed of α_S- and β-caseins. The composition of these aggregates changed with the application of shear, suggesting the occurrence of orthokinetic aggregation and fragmentation [11]. Apart from that, whey proteins are also known to undergo shear-induced structural transformations, resulting in the unfolding and propagation of interactions that lead to aggregation within the milk system [5].

These aforementioned findings suggest that shear application in conjunction with other factors has the potential to modulate the structural properties of MPC dispersions. While previous research has explored the effects of pH and shear on MPCs, there remains a significant gap in the literature regarding the combined influence of these factors during heat treatment, particularly at the pH levels commonly encountered in various dairy applications ranging from pH 6.1 to 7.5. Therefore, studying the combined effects of heat and shear in a pH-modified environment can provide valuable insights into protein–protein interactions and their potential implications. The present study focused on investigating the influence of pH (6.1, 6.4, 6.8, or 7.5) and shear (0, 100, or 1000 s^{-1}) on heat-induced (90 °C for 5 min and 121 °C for 2.6 min) changes in milk proteins in 8% MPC suspensions.

2. Materials and Methods

2.1. Materials

MPC powder was obtained from Fonterra Co-operative (Palmerston North, New Zealand). The composition of the MPC powder, according to the manufacturer's specifications, was 81.0% (*w*/*w*) total protein, 1.6% (*w*/*w*) fat, 5.5% (*w*/*w*) carbohydrate, and 7.2% (*w*/*w*) ash. Chemicals for analysis were purchased from Sigma-Aldrich Pty Ltd., based in Castle Hill, NSW, Australia, and ultrapure water (Milli-Q water, Merck Millipore, Bayswater, VIC, Australia) was consistently used throughout the study.

2.2. Sample Preparation and Treatment

MPC suspensions were prepared by reconstituting MPC powder in Milli-Q water, achieving a pH of 6.8 after reconstitution. The solutions were stirred continuously at 50 °C for 1 h to ensure complete solubilization of the powder, followed by overnight storage at 4 °C for further hydration [12]. The next day, samples were allowed to equilibrate at 25 °C for 1 h and the pH of the MPC suspensions was adjusted to 6.1, 6.4, 6.8, or 7.5 with concentrated HCl or NaOH. The final volume was adjusted using Milli-Q water to obtain a final protein concentration of 8% (*w*/*w*).

The MPC suspensions were subjected to heat treatment of 90 °C for 5 min or 121 °C for 2.6 min. A constant shear rate of either 100 or 1000 s^{-1} was maintained inside a pressure cell (CC25/PR-150) of a rheometer (Physica MCR 301 series, Anton Paar GmbH, Ostfildern-Scharnhausen, Germany). A constant pressure of 250 kPa was maintained throughout the experiments. This pressure is crucial for eliminating air bubbles, enhancing sample-to-measuring surface contact, stabilizing the sample during testing, ensuring consistent

experimental conditions, and preventing evaporation when heating the samples, thereby facilitating accurate and reproducible rheological measurements across various samples. Samples subjected to heating under two temperatures were heated at a rate of 5 °C min^{-1} to the targeted temperature and held there for the required time and cooled at a rate of 5 °C min^{-1}.

The pH of each sample was measured immediately after treatment using a pH meter (WTW Inolab pH 720, Weilheim, Germany).

After processing, the samples were separated via sequential centrifugation using a Beckman L-70 Ultracentrifuge equipped with a Type 70.ITI rotor (Beckman Instruments, Inc., Brea, CA, USA) at 20 °C at 5700 or 75,940× *g* for 1 h, following a method previously described [13]. The resulting supernatants were collected for protein profile analysis. The protein fraction in the supernatants after centrifugation at 75,940 g was classified as "non-sedimentable". The proteins that were sedimented following the 5700 g centrifugation were classified as "aggregated".

2.3. Particle Size and Zeta Potential Measurements

Particle size and zeta potential measurements were conducted immediately after each treatment as described previously [14]. The measurements were performed using a Zetasizer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). Prior to the measurements, the treated samples were diluted 1000 times using skim milk ultra-filtrate. In the calculations, the refractive indexes of 1.57 and 1.38 were used for MPC and skim milk ultra-filtrate, respectively.

2.4. Fourier Transform Infrared (FTIR) Analysis

The assessment of changes in the secondary structure of proteins was conducted using an FTIR spectrometer (PerkinElmer Frontier FTIR Spectrometer, Boston, MA, USA) as described previously [14]. FTIR spectra were acquired at room temperature (~20 °C) within 10 min after each treatment. Each spectrum was an average of 16 scans with a resolution of 4 cm^{-1} after subtracting the background. To enhance the resolution for qualitative analysis, the second derivative of all FTIR spectra was obtained within the broad amide I region of 1700–1600 cm^{-1}. The obtained spectra were processed using Fourier self-deconvolution (FSD) and baseline correction with Origin Student 2019b software (Origin Lab Corporation, Northampton, MA, USA). The areas of the prominent peaks assigned to specific secondary structures were summed up and divided by the total area, resulting in the identification of five major peak areas corresponding to protein secondary structures, including intramolecular β sheets (1637–1615 cm^{-1}), aggregated β sheets (1700–1682 cm^{-1}), random coils (1645–1638 cm^{-1}), α-helices (1664–1646 cm^{-1}), and β turns (1681–1665 cm^{-1}). The obtained results were then subjected to statistical analysis following the guidelines outlined in Section 2.6.

2.5. Reverse Phase-High Performance Liquid Chromatography Analysis

Whole samples and supernatants of each centrifuged MPC suspension were analysed for the content of α_{S1}-casein (α_{S1}-CN), α_{S2}-casein (α_{S2}-CN), β-casein (β-CN), κ-casein (κ-CN), α-lactalbumin (α-LA), and β-lactoglobulin (β-LG) by RP-HPLC using a Zorbax 300SB-C8 RP-HPLC column (silica-based packing, 3.5 micron, 300A, Agilent Technologies Inc., Mulgrave VIC, Australia) as the stationary phase. Water (mobile phase A) and acetonitrile (mobile phase B) solutions both containing 0.1% (*v*/*v*) trifluoroacetic acid (TFA) were used as mobile phases. A gradient elution was run at a constant flow rate of 0.8 mL/min as described previously [15].

2.6. Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics software (version 28.0.1.0, IBM Corp., Armonk, NY, USA) employing a general linear model (GLM) approach. The study was arranged as a randomized block, full factorial design with pH as the main plot,

while the subplots were temperature/time combinations and shearing. This block was replicated at least twice, with each replication consisting of three sub-samplings. The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Particle Size Distribution and Zeta Potential of MPC Suspensions

In unheated MPC suspensions, the largest particles (~235 nm) were observed in the sample at pH 6.1 and the lowest particle size at pH 6.8 (~207 nm) (Figure 1 and Table 1).

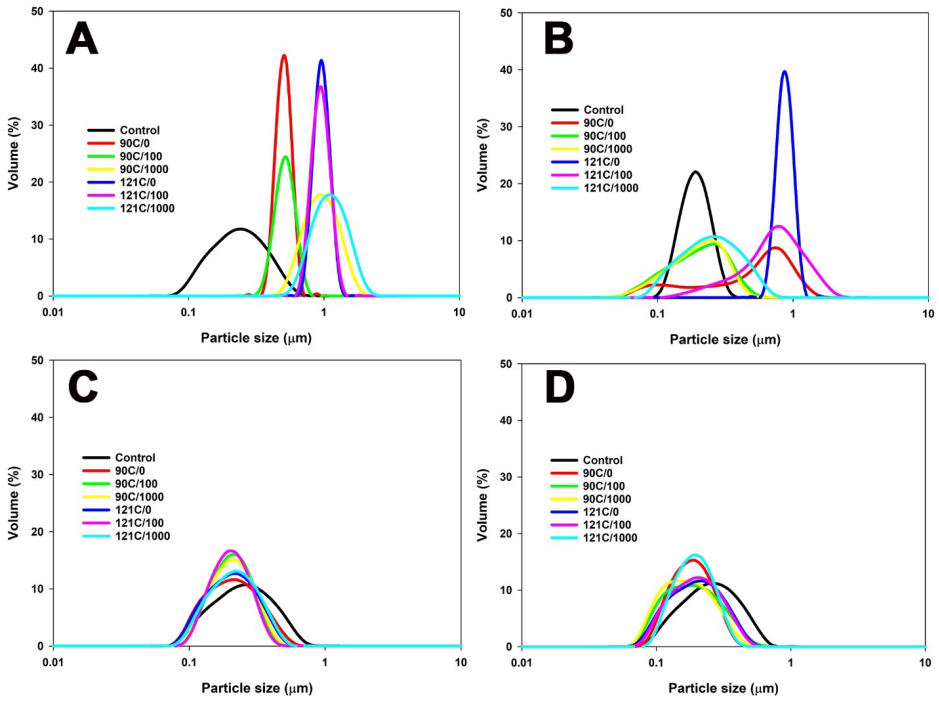

Figure 1. Particle size distribution of milk protein suspensions with pH adjusted to 6.1 (**A**), 6.4 (**B**), 6.8 (**C**), or 7.5 (**D**) and sheared at 100 s^{-1} or 1000 s^{-1} during heating at 90 °C for 5 min or 121 °C for 2.6 min. Please note that in the figure labels, 'C' represents degrees Celsius (°C), and the numerical value following the slash denotes shear rate per second (s^{-1}). The true controls were assessed prior to heating without shear (0 s^{-1}) at their respective pH.

Heating the MPC suspensions with initial pH values of 6.1 or 6.4 led to an increase in particle size and these were larger at pH 6.1 than at pH 6.4 (Figure 1 and Table 1); the extent of these increases was far larger than what would be expected from whey protein denaturation [16,17] and suggests it to be due to casein micelle aggregation. In contrast, at higher pH (≥pH 6.8), heating consistently reduced particle size, indicating a tendency towards the formation of smaller aggregates or the dissociation of casein micelles. Heating the MPC suspensions without shear at pH 6.8 caused a reduction in particle size, from ~207 nm at 20 °C, to ~182 nm at 90 °C, and ~179 nm at 121 °C (Figure 1 and Table 1). This decrease may be attributed to the heat-induced dissociation of casein micelles or the formation of smaller whey protein aggregates [18]. A similar trend was observed in the MPC suspension with a pH of 7.5, showing a reduction in particle size from ~214 nm at 20 °C, to ~196 and ~184 nm after heating at 90 and 121 °C, respectively. This decrease can be mainly attributed to the formation of smaller soluble aggregates [19].

The application of shear, in conjunction with heat, further influenced these trends. At pH 6.1, the combined application of heat and shear resulted in an increase in particle size, particularly at 121 °C and 1000 s^{-1} (Table 1). This is in contrast to the behaviour observed at pH 6.4, where similar conditions led to a significant decrease in particle size with an increasing shear rate. At pH 6.8, the combined application of heat and shear did not change

the particle size. However, at pH 7.5, a consistent trend of particle size reduction under the influence of heat and shear was observed, with the smallest particle size observed at a shear rate of 1000 s^{-1} at 121 °C.

Table 1. Average particle size of MPC suspensions with pH adjusted to 6.1, 6.4, 6.8, or 7.5 and subjected to different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

pH	Temperature (°C)	Particle Size (nm)		
		0 s^{-1}	**100 s^{-1}**	**1000 s^{-1}**
6.1	20	235 ^{E}		
	90	714 ^{Cc}	850 ^{Bb}	1974 ^{Ba}
	121	1632 ^{Ac}	1842 ^{Ab}	2132 ^{Aa}
6.4	20	224 ^{F}		
	90	384 ^{Da}	330 ^{Dc}	338 ^{Db}
	121	1146 ^{Ba}	796 ^{Cb}	498 ^{Cc}
6.8	20	207 ^{H}		
	90	182 ^{Ja}	180 ^{Ea}	178 ^{Ea}
	121	179 ^{Ja}	178 ^{Ea}	177 ^{Ea}
7.5	20	214 ^{G}		
	90	196 ^{Ia}	174 ^{Eb}	166 ^{Fc}
	121	184 ^{Ja}	180 ^{Ea}	170 ^{Fb}
SEM_{pooled} *		0.675		

Different uppercase letter superscripts indicate a significant difference among the means within a column, while lowercase superscripts signify a significant difference among the means within a raw; $p < 0.05$. * Pooled Standard Error of Mean.

At 20 °C, a zeta potential of ~−18 mV was observed in MPC suspensions at both pH 6.1 and 6.4. Increasing the pH to 6.8 and 7.5 led to a rise in the zeta potentials of ~−21 mV and −25 mV, respectively, highlighting a pH-dependent trend in the increase in negative zeta potential. However, heating did not notably affect the zeta potential of the MPC suspensions at different pH levels (Table 2).

Table 2. Zeta potential of MPC suspensions with pH adjusted to 6.1, 6.4, 6.8, or 7.5 and subjected to different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

pH	Temperature (°C)	Zeta Potential (mV)		
		0 s^{-1}	**100 s^{-1}**	**1000 s^{-1}**
6.1	20	−18.4 ^{AB}		
	90	−19.0 ^{ABa}	−19.0 ^{BCa}	−20.2 ^{ABa}
	121	−19.8 ^{ABa}	−18.4 ^{Aba}	−16.5 ^{Ab}
6.4	20	−18.6 ^{AB}		
	90	−17.8 ^{Aa}	−17.5 ^{Aa}	−18.9 ^{ABb}
	121	−18.3 ^{Aa}	−19.5 ^{BCb}	−20.5 ^{ABc}
6.8	20	−21.2 ^{C}		
	90	−21.2 ^{Ca}	−21.2 ^{Da}	−21.1 ^{Ba}
	121	−20.8 ^{Ca}	−19.9 ^{CDa}	−21.1 ^{Ba}
7.5	20	−25.3 ^{D}		
	90	−26.0 ^{Da}	−26.4 ^{Ea}	−26.8 ^{Ca}
	121	−25.1 ^{Da}	−25.9 ^{Ea}	−25.5 ^{Ca}
SEM_{pooled} *		0.419		

Different uppercase letter superscripts indicate a significant difference among the means within a column, while lowercase superscripts signify a significant difference among the means within a raw; $p < 0.05$. * Pooled Standard Error of Mean.

The application of shear during heating significantly ($p < 0.05$) influenced the zeta potential, particularly at lower pH values. For example, when MPC suspensions at pH 6.1 were heated at 121 °C and sheared at 1000 s^{-1}, the zeta potential changed from ~−20 mV

in the absence of shear to ~−17 mV. Conversely, at 90 °C, the zeta potential at pH 6.1 remained consistent across different shear rates, indicating that, at this temperature, shear forces did not significantly influence the zeta potential. In contrast, at pH 6.4, the combined application of heat and shear resulted in an increased zeta potential at both 90 °C and 121 °C compared to the suspensions at 20 °C. The increase was more pronounced at 121 °C, where the zeta potential rose to ~−20.5 mV at 1000 s^{-1}, from ~−18.6 mV at 20 °C. The combined application of heat and shear did not result in any significant changes in the zeta potential of MPC suspensions at pH 6.8 and 7.5 (Table 2).

3.2. Partitioning of Proteins in MPC Suspensions

3.2.1. Changes in Protein Content within the Non-Sedimentable Fraction

In the unheated sample at pH 6.8, the non-sedimentable (75,490× *g* for 60 min at 20 °C) fraction contained ~15% of total α_{S1}-CN, ~20% of total α_{S2}-CN, ~20% of total β-CN, and ~28% of total κ-CN (Table 3), as well as most of the β-LG (~97%) and α-LA (~98%) (Table 3).

Table 3. Proportion of α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, β-LG, and α-LA in the non-sedimentable fraction (75,490× *g* for 1 h at 20 °C), expressed as a percentage of their content in the original bulk unheated suspensions with pH adjusted to 6.8, subjected to different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

pH	Temperature (°C)	Shear Rate (s^{-1})	% Protein Content in Non-Sedimentable Fraction					
			α_{s1}-CN	**α_{s2}-CN**	**β-CN**	**κ-CN**	**β-LG**	**α-LA**
6.1	20	0	2.4 ^{g}	3.6 ^{h}	5.0 ^{j}	12.5 ^{k}	70.5 ^{c}	76.0 ^{fgh}
	90	0	0.0	0.0	0.0	6.0 ^{m}	41.7 ^{i}	52.4 ^{kl}
	90	100	0.0	0.0	0.0	4.5 ^{m}	30.0 ^{j}	20 ^{n}
	90	1000	0.0	0.0	0.0	3.0 ^{m}	12.0 ^{l}	11.1 ^{o}
	121	0	0.0	0.0	0.0	4.3 ^{m}	23.3 ^{k}	39.4 ^{m}
	121	100	0.0	0.0	0.0	4.7 ^{m}	22.2 ^{k}	16.7 ^{n}
	121	1000	0.0	0.0	0.0	3.0 ^{m}	20 ^{k}	8.0 ^{o}
6.4	20	0	4.3 ^{g}	9.3 ^{g}	8.3 ^{ij}	12.5 ^{k}	85.0 ^{b}	81.3 ^{de}
	90	0	0.0	1.7 ^{h}	2.7 ^{j}	12.1 ^{k}	52.8 ^{gh}	65.1 ^{j}
	90	100	0.0	13.6 ^{g}	8.6 ^{ij}	40.5 ^{hi}	57.3 ^{fg}	71.8 ^{hi}
	90	1000	0.0	10.7 ^{g}	11.4 ^{i}	62.5 ^{cd}	68.6 ^{c}	81.9 ^{de}
	121	0	0.0	0.0	0.0	7.1 ^{lm}	41.5 ^{i}	57.5 ^{k}
	121	100	0.0	0.0	0.0	25.0 ^{j}	53.3 ^{gh}	65.5 ^{j}
	121	1000	0.0	0.0	0.0	36.4 ^{i}	65.6 ^{cde}	73.1 ^{ghi}
6.8	20	0	15.0 ^{f}	20.0 ^{f}	20.0 ^{h}	28.0 ^{j}	97.0 ^{a}	98.0 ^{a}
	90	0	16.5 ^{ef}	24.8 ^{def}	28.7 ^{fg}	36.2 ^{i}	51.1 ^{h}	68.0 ^{ij}
	90	100	21.1 ^{cde}	22.5 ^{ef}	33.7 ^{def}	46.7 ^{fg}	66.3 ^{cd}	76.1 ^{fgh}
	90	1000	22.6 ^{bcd}	25.0 ^{d}	41.8 ^{b}	63.0 ^{cd}	70.0 ^{c}	77.0 ^{efg}
	121	0	20.5 ^{cdef}	29.4 ^{cd}	35.5 ^{cde}	42.3 ^{gh}	40.5 ^{i}	49.6 ^{l}
	121	100	23.5 ^{bcd}	34.0 ^{bc}	53.3 ^{a}	56.5 ^{e}	60.4 ^{efg}	71.6 ^{hi}
	121	1000	23.8 ^{bcd}	34.4 ^{bc}	57.0 ^{a}	57.3 ^{e}	62.9 ^{def}	77.5 ^{efg}
7.5	20	0	21.3 ^{cde}	21.7 ^{ef}	26.4 ^{g}	49.5 ^{f}	81.9 ^{b}	90.5 ^{b}
	90	0	19.8 ^{def}	25.0 ^{def}	31.0 ^{efg}	58.2 ^{de}	53.3 ^{gh}	81.8 ^{de}
	90	100	25.0 ^{bc}	25.9 ^{de}	40.0 ^{bc}	65.6 ^{c}	68.9 ^{c}	85.4 ^{bcd}
	90	1000	33.3 ^{a}	38.7 ^{b}	42.7 ^{b}	73.7 ^{b}	67.1 ^{cd}	89.8 ^{bc}
	121	0	26.8 ^{b}	27.8 ^{d}	38.8 ^{bcd}	63.4 ^{c}	42.9 ^{i}	68.9 ^{ij}
	121	100	22.2 ^{bcd}	42.1 ^{a}	54.7 ^{a}	81.6 ^{a}	51.7 ^{h}	78.6 ^{ef}
	121	1000	36.2 ^{a}	43.9 ^{a}	56.7 ^{a}	85.9 ^{a}	54.9 ^{gh}	82.9 ^{cde}
SEM_{pooled} *			0.143	0.034	0.210	0.085	0.120	0.097

Different lowercase letter superscripts indicate a significant difference among the means within a column ($p < 0.05$). * Pooled Standard Error of Mean.

Lowering the pH resulted in a notable decrease in the content of both caseins and whey proteins in the non-sedimentable fraction. Specifically, at pH 6.4 and further at 6.1,

the κ-CN content decreased to ~13% of its total, alongside reductions in α_{S1}-CN, α_{S2}-CN, and β-CN levels. Moreover, this pH adjustment led to a more pronounced decrease in β-LG and α-LA levels, with the most significant reduction occurring at pH 6.1, where β-LG and α-LA levels decreased by >10% compared to those at pH 6.4. On the other hand, an increase in pH to 7.5 resulted in an increase in non-sedimentable κ-CN, up to ~50% of its total, as well as increased levels of non-sedimentable α_{S1}-CN, α_{S2}-CN, and β-CN (Table 3).

At pH 6.8, heat treatment alone resulted in an increase in levels of non-sedimentable κ-CN, reaching ~42% of the total κ-CN at 121 °C (Table 3). The levels of non-sedimentable β-CN, α_{S1}-CN, and α_{S2}-CN also increased. Simultaneously, both β-LG and α-LA declined with β-LG showing a more pronounced decrease by ~41% at 121 °C. At pH < 6.8, heating further reduced both the casein and whey protein levels in the non-sedimentable fraction compared to non-heated suspensions.

At pH 6.4 or 6.1, heating at 121 °C without shear resulted in a notable reduction in non-sedimentable κ-CN, while the other caseins were not found in the non-sedimentable phase. This was also accompanied by a decrease in non-sedimentable β-LG and α-LA (Table 3). At pH 7.5, heat treatment further increased non-sedimentable κ-CN levels, up to ~63% of total κ-CN at 121 °C. Additionally, an increase in β-CN (~12% of the total at 121 °C) and slight increases in α_{S1}-CN and α_{S2}-CN (~6% at 121 °C) were also noted at this pH. Non-sedimentable β-LG and α-LA also declined at this pH with an effect more pronounced for β-LG.

The combined application of heat and shear, along with an increase in pH, notably enhanced the levels of all caseins, particularly κ-CN, in the non-sedimentable fraction. For example, at pH 6.8, non-sedimentable κ-CN increased, e.g., by ~15% of total κ-CN at 121 °C when the shear rate was changed from 0 s^{-1} to 1000 s^{-1} (Table 3). Levels of non-sedimentable α_{S1}-CN, α_{S2}-CN, and β-CN also showed direct shear dependency as their concentration rose at both temperatures and pH 6.8, with a more notable rise observed in β-CN, reaching up to ~57% of total β-CN (Table 3). Furthermore, at these conditions at pH 6.8, non-sedimentable β-LG and α-LA also increased by ~22% and ~28% of their totals, respectively, at 121 °C and a shear rate of 1000 s^{-1} (Table 3).

The combined application of heat and shear elevated the levels of both casein and whey proteins in the non-sedimentable phase as the pH increased. At pH 6.1, the level of caseins within the non-sedimentable fraction was unaffected by the combined application of heat and shear. However, the applied shear resulted in a decrease in both β-LG and α-LA (Table 3). At pH 6.4, the combined application of heat and shear resulted in an increase in non-sedimentable κ-CN content at both temperatures (Table 3), e.g., by ~29% at 121 °C and a shear rate of 1000 s^{-1}. No distinct trend could be observed for α_{S1}-CN while both α_{S2}-CN and β-CN experienced slight increases. Under these conditions, both β-LG and α-LA concentrations in the non-sedimentable fraction increased. The impact was more prominent on β-LG with a 24% increase at 121 °C and at 1000 s^{-1} compared to 16% in α-LA under the same conditions.

At pH 7.5, the combined application of heat and shear significantly increased the κ-CN content, reaching ~86% of the total at 121 °C and a shear rate of 1000 s^{-1} (Table 3). Additionally, the levels of non-sedimentable α_{S1}-CN, α_{S2}-CN, and β-CN also increased. For instance, at 121 °C and a shear rate of 1000 s^{-1}, the concentration of α_{S1}-CN, α_{S2}-CN, and β-CN increased by ~9, ~16, and ~18%, respectively. Furthermore, both β-LG and α-LA levels were also elevated. Specifically, β-LG and α-LA contents increased by ~12 and ~14%, respectively, at 121 °C and a shear rate of 1000 s^{-1}.

3.2.2. Changes in the Content of Aggregated Protein

In the unheated sample at pH 6.8, the aggregated fraction, defined as the fraction that had sedimented after centrifugation at 5700× *g* for 60 min at 20 °C, contained ~5% of total α_{S1}-CN, ~7% of total α_{S2}-CN, ~8% of total β-casein, and ~10% of total κ-CN (Table 3), and very little β-LG (~2%) and α-LA (~1%) (Table 4). Lowering the pH increased both caseins and whey proteins within the aggregated fraction with a pronounced increase observed

at pH 6.1. Decreasing the pH to 6.4 or 6.1 resulted in a substantial increase in sedimented κ-CN, reaching ~36% and ~53%, respectively (Table 4).

Table 4. Content of α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, β-LG, and α-LA in the sedimentable fraction (5700 g for 60 min at 20 °C) as a percentage of their content in the original bulk unheated suspensions with pH adjusted to 6.8, subjected to different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

pH	Temperature (°C)	Shear Rate (s^{-1})	% Protein Content in Sedimentable Fraction					
			α_{s1}-CN	α_{s2}-CN	β-CN	κ-CN	β-LG	α-LA
6.1	20	0	85.7 ^{b}	81.0 ^{c}	80.0 ^{d}	52.5 ^{d}	14.1 ^{ij}	15.7 ^{gh}
	90	0	99.8 ^{a}	89.7 ^{b}	85.0 ^{c}	68.8 ^{c}	43.3 ^{d}	37.5 ^{e}
	90	100	100.0 ^{a}	98.5 ^{a}	96.0 ^{a}	73.2 ^{c}	55.0 ^{c}	55.0 ^{c}
	90	1000	100.0 ^{a}	99.2 ^{a}	98.0 ^{a}	80.0 ^{b}	68.7 ^{b}	61.1 ^{b}
	121	0	98.5 ^{a}	99.7 ^{a}	90.0 ^{b}	78.3 ^{b}	52.2 ^{c}	46.2 ^{d}
	121	100	100.0 ^{a}	100.0 ^{a}	98.0 ^{a}	80.0 ^{b}	64.4 ^{b}	58.3 ^{b}
	121	1000	100.0 ^{a}	100.0 ^{a}	100.0 ^{a}	85.0 ^{a}	74.0 ^{a}	67.0 ^{a}
6.4	20	0	61.3 ^{e}	41.9 ^{f}	58.3 ^{g}	36.3 ^{e}	2.0 ^{k}	4.4 ^{jkl}
	90	0	75.7 ^{d}	75.9 ^{d}	64.8 ^{f}	50.0 ^{d}	22.3 ^{fg}	11.8 ^{hi}
	90	100	80.8 ^{c}	72.7 ^{d}	68.5 ^{f}	24.3 ^{gh}	17.4 ^{ghi}	9.7 ^{ij}
	90	1000	79.1 ^{cd}	71.4 ^{d}	70.5 ^{ef}	12.5 ^{i}	10.4 ^{j}	5.5 ^{jkl}
	121	0	98.7 ^{a}	81.0 ^{c}	70.0 ^{ef}	73.8 ^{c}	31.4 ^{e}	27.5 ^{f}
	121	100	100.0 ^{a}	73.5 ^{d}	73.6 ^{e}	33.3 ^{ef}	22.9 ^{fg}	18.5 ^{g}
	121	1000	100.0 ^{a}	62.5 ^{e}	75.0 ^{e}	30 ^{f}	11.1 ^{j}	11.6 ^{hi}
6.8	20	0	5.0 ^{h}	7.0 ^{l}	8.0 ^{k}	10.0 ^{ij}	2.0 ^{k}	1.0 ^{l}
	90	0	19.3 ^{g}	23.9 ^{gh}	18.4 ^{i}	25.3 ^{g}	21.1 ^{fgh}	15.1 ^{gh}
	90	100	7.8 ^{h}	19.1 ^{hi}	14.1 ^{ij}	18.5 ^{h}	10.5 ^{j}	7.6 ^{ijk}
	90	1000	5.4 ^{h}	15.2 ^{ij}	8.8 ^{k}	10.0 ^{ij}	10.0 ^{j}	12.0 ^{hi}
	121	0	25.1 ^{f}	28.2 ^{g}	25.6 ^{h}	35.6 ^{e}	24.8 ^{f}	19.6 ^{g}
	121	100	5.1 ^{h}	5.3 ^{l}	2.2 ^{l}	23.5 ^{gh}	8.8 ^{j}	7.4 ^{ijk}
	121	1000	3.2 ^{i}	5.8 ^{l}	1.1 ^{l}	19.8 ^{h}	9.3 ^{j}	9.0 ^{ij}
7.5	20	0	6.1 ^{h}	9.6 ^{k}	8.3 ^{k}	10.8 ^{ij}	4.2 ^{k}	3.6 ^{k}
	90	0	7.7 ^{h}	14.3 ^{ijk}	18.4 ^{i}	13.8 ^{i}	18.0 ^{ghi}	3.4 ^{k}
	90	100	4.5 ^{h}	12.9 ^{jk}	16.7 ^{i}	2.1 ^{k}	9.5 ^{j}	2.4 ^{k}
	90	1000	4.6 ^{h}	9.9 ^{kl}	10.6 ^{jk}	4.0 ^{k}	10.0 ^{hi}	2.5 ^{k}
	121	0	8.5 ^{h}	16.5 ^{ij}	25.5 ^{h}	13.5 ^{i}	16.5 ^{hi}	1.3 ^{l}
	121	100	7.8 ^{h}	7.4 ^{l}	17.4 ^{i}	6.2 ^{jk}	13.3 ^{ij}	1.4 ^{l}
	121	1000	5.3 ^{h}	7.1 ^{l}	11.1 ^{jk}	2.1 ^{k}	10.9 ^{j}	0.9 ^{l}
SEM_{pooled} *			0.093	0.125	0.115	0.145	0.085	0.098

Different lowercase letter superscripts indicate a significant difference among the means within a column ($p < 0.05$). * Pooled Standard Error of Mean.

In addition, the levels of α_{S1}-CN, α_{S2}-CN, and β-CN appeared to be pH-dependent as their content in the aggregated fraction was greatest at pH 6.1, up to ~80% of their total. Lowering the pH to 6.4 or 6.1 also resulted also in a more pronounced increase in β-LG and α-LA levels in the aggregated fractions. Increasing pH to 7.5 did not affect the levels of caseins in this fraction noticeably, with only a slight increase observed in both β-LG and α-LA.

Heat treatment without shear increased κ-CN levels in the aggregated fraction (e.g., to ~36% of the total after heat treatment at 121 °C) at pH 6.8 (Table 4). This was also accompanied by a rise in the levels of aggregated β-CN, α_{S1}-CN, and α_{S2}-CN (Table 4). Both β-LG and α-LA levels in the aggregated fraction were also greater, with a comparatively greater increase observed for β-LG, especially at 121 °C, where ~25% of the total β-LG and 20% of the total α-LA were observed. Reducing the pH to 6.4 or 6.1 led to a notable increase in all caseins in the aggregated fraction. Additionally, the levels of β-LG and α-LA also increased, particularly more at pH 6.1, reaching ~52% and ~46%,

respectively (Table 4). At pH 7.5, heating of MPC suspensions did not change the levels of κ-CN and α_{S1}-CN in the aggregated fraction, but β-CN and α_{S2}-CN levels increased by ~12% and ~6%, respectively, at 121 °C. This pH also affected the behaviour of α-LA and β-LG, with only β-LG rising up to ~17% at 121 °C in the aggregated fraction (Table 4).

The combined application of heat and shear showed that the protein composition of the aggregated fraction was clearly pH dependent—higher pH at heating and shearing resulted in less incorporation of caseins and whey proteins into the aggregates. For example, a small proportion of the total caseins was incorporated at pH 7.5 in comparison to those at other pH levels (Table 4). κ-CN appeared to be affected the most as its levels in the aggregates rose as the pH was lowered. In general, higher shear reduced the levels of caseins in the aggregates during heating (Table 4). However, this trend was somewhat reversed when pH was reduced to 6.1. Most caseins were incorporated in these aggregates, even in the absence of shear at this pH, and shear had no observable effect. The level of κ-CN increased from that without shear, indicating greater incorporation of this protein under the shear (Table 4). This, however, was the opposite to its levels at pH 6.4 as the level of κ-CN declined upon shearing indicating that electrostatic interactions may have governed some of the aggregation that could have been manipulated by shearing to a certain effect.

β-LG and α-LA followed a similar pattern to that of κ-CN as their levels in the aggregated fraction were clearly affected by pH and shear (Table 4). Their incorporation was minimal at pH 7.5 and, even then, it was further reduced by greater shearing. As pH was adjusted to 6.8 or 6.4, more and more of these proteins appeared to be incorporated into the aggregates. In these cases, the extent of shear was inversely related as it reduced their levels substantially at the greater shear, almost by 60% in comparison to those in the absence of shear. This effect, however, was reversed when the samples were heated and sheared at pH 6.1. The levels of β-LG and α-LA in this case increased by an average of 42–44% in comparison to these in the aggregates obtained under quiescent conditions (Table 4).

3.3. Conformational Properties of Proteins in MPC Suspensions

The proportion of secondary structures in the unheated samples depended on pH (Table 5). The secondary structure in the control unheated MPC suspensions was mainly intramolecular β-sheets accounting for ~45% of the total (Table 5). Adjusting the pH to 6.4 or 6.1 at 20 °C reduced the intramolecular β-sheets and α-helical structures with a more pronounced effect at pH 6.1 (Figure 2 and Table 5).

Increasing pH to 7.5 led to a decrease in intramolecular β-sheets, up to ~40%, likely due to the disruption of native conformation of whey proteins and an increase in random structures due to changes in protein interactions, and this led to destabilisation of the micellar structure [20].

In the MPC suspensions at pH 6.8, heating resulted in a pronounced decrease in intramolecular β-sheet structures (Figure 2 and Table 5) combined with an increase in β-turn structures (Figure 2). Heating MPC suspensions without shear at pH 6.4 or 6.1 further decreased intramolecular β-sheets and α-helix structures (Table 5). At pH 7.5, heating resulted in a further decline in the content of intramolecular β-sheets and β-turns, accompanied by a prominent increase in the content of random structures.

The concurrent application of heat and shear to MPC suspensions at pH 6.8 further reduced the intramolecular β-sheet structures while elevating the presence of β-turns. Additionally, a decrease in α-helix content and a simultaneous rise in random coils were observed (Figure 2 and Table 5). Moreover, the aggregated β-sheet structures also exhibited a decrease compared to heated dispersions. In the MPC suspensions with pH adjusted to pH 6.4 and 6.1, a shear-dependent reduction in intramolecular β-sheets and α-helical content was observed at both temperatures (Figure 2 and Table 5). At pH 7.5, the combined application of heat and shear decreased the content of α-helix structures compared to heated suspensions, with a more pronounced effect at 121 °C and at 1000 s^{-1}.

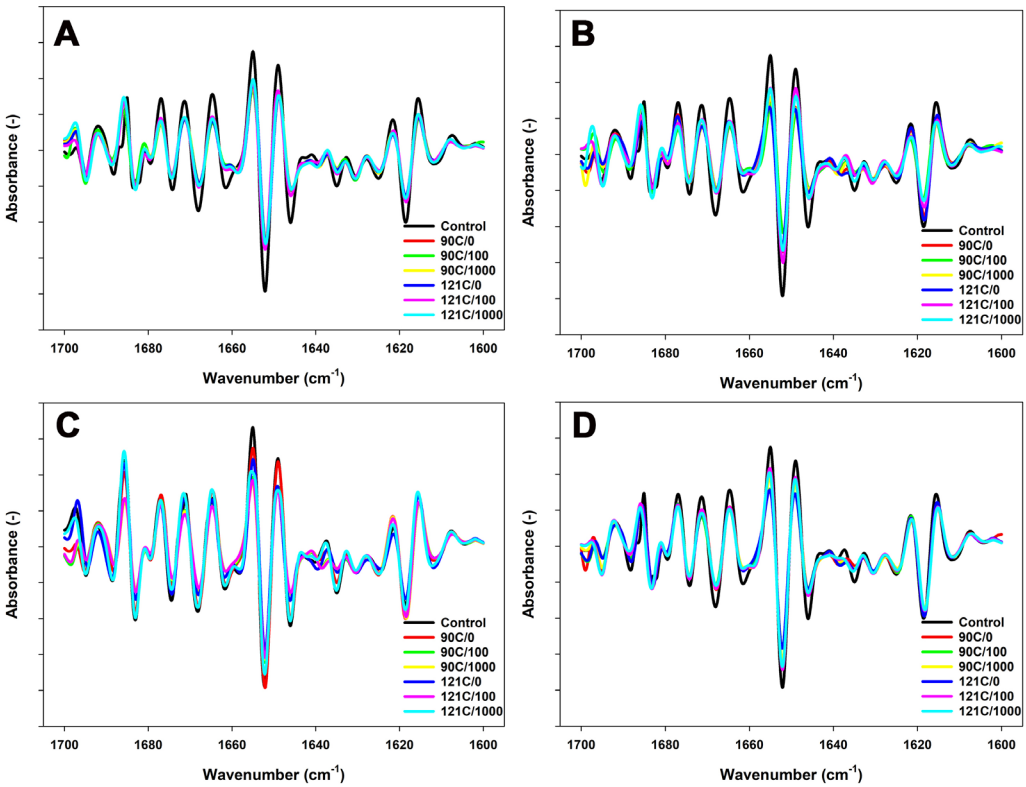

Figure 2. FTIR spectra (second derivative) for Amide I region of MPC dispersions with pH adjusted to 6.1 (**A**), 6.4 (**B**), 6.8 (**C**), or 7.5 (**D**) and sheared at 100 s^{-1} or 1000 s^{-1} during heating at 90 °C for 5 min or 121 °C for 2.6 min. Please note that in the figure labels. 'C' represents degrees Celsius (°C), and the numerical value following the slash denotes the applied shear rate per second (s^{-1}). The true controls were assessed prior to heating without shear (0 s^{-1}) at their respective pH.

Table 5. Total percentage areas of different secondary structures in Amide I region of proteins milk dispersions with pH adjusted to 6.1, 6.4, 6.8, or 7.5 and subjected to different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

pH	Temp (°C)	Intramolecular β-Sheets (1615–1637)			Random Coils (1638–1645)			α-Helix (1646–1664)			β-Turns (1665–1681)			Aggregated β-Sheets (1682–1700)		
		0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}
6.1	20	31.85 ^{EF}			4.31 ^{F}			18.31 ^{E}			35.14 ^{C}			10.39 ^{B}		
	90	28.70 ^{Ga}	21.97 ^{Eb}	18.72 ^{Db}	5.11 ^{Fc}	10.35 ^{Fb}	12.81 ^{Ga}	15.49 ^{Fa}	10.51 ^{Eb}	6.53 ^{Dc}	37.19 ^{Bc}	38.83 ^{Bb}	41.81 ^{Ba}	13.51 ^{Ac}	18.34 ^{Ab}	20.13 ^{Ba}
	121	23.01 ^{Ha}	19.88 ^{Fb}	10.94 ^{Eb}	5.25 ^{Fc}	6.88 ^{Gb}	11.53 ^{Ga}	10.51 ^{Ga}	6.82 ^{Fb}	5.99 ^{Db}	47.65 ^{Aa}	48.55 ^{Aa}	49.01 ^{Aa}	13.58 ^{Ac}	17.87 ^{Ab}	22.53 ^{Aa}
6.4	20	36.71 ^{C}			4.17 ^{F}			21.15 ^{CD}			29.14 ^{E}			8.83 ^{C}		
	90	33.11 ^{DEa}	29.42 ^{Cb}	24.43 ^{Cc}	5.15 ^{Fc}	11.11 ^{Fb}	18.35 ^{Fa}	17.30 ^{Ea}	12.81 ^{Db}	10.30 ^{Cc}	33.93 ^{Da}	32.53 ^{Da}	30.81 ^{Cb}	10.51 ^{Bc}	14.13 ^{Bb}	16.11 ^{Ca}
	121	30.56 ^{Fa}	27.44 ^{Db}	24.32 ^{Cc}	7.28 ^{Ec}	14.28 ^{Eb}	22.38 ^{Ea}	11.53 ^{Ga}	10.83 ^{Eab}	9.95 ^{Cc}	37.80 ^{Ba}	35.21 ^{Cb}	30.82 ^{Dc}	12.83 ^{Aa}	12.24 ^{Ca}	12.53 ^{Da}
6.8	20	45.01 ^{A}			5.22 ^{F}			27.01 ^{A}			19.21 ^{H}			3.55 ^{G}		
	90	40.77 ^{Ba}	27.40 ^{Db}	24.19 ^{Cc}	8.31 ^{Ec}	20.15 ^{Db}	27.18 ^{Da}	21.59 ^{Ca}	20.31 ^{Aa}	14.83 ^{ABb}	23.50 ^{Gc}	27.32 ^{Eb}	30.52 ^{Ca}	5.83 ^{DEa}	4.82 ^{Fab}	3.28 ^{Fb}
	121	36.07 ^{Ca}	14.96 ^{Gb}	11.53 ^{Ec}	19.65 ^{Cc}	22.51 ^{Cb}	30.04 ^{Ca}	10.31 ^{Gb}	18.51 ^{Ba}	11.35 ^{Cb}	27.0 ^{Fc}	38.51 ^{Bb}	42.81 ^{Ba}	6.97 ^{Da}	5.51 ^{Fab}	4.27 ^{Fb}
7.5	20	40.04 ^{B}			12.54 ^{D}			25.51 ^{B}			17.38 ^{I}			4.53 ^{EF}		
	90	37.53 ^{Ca}	33.28 ^{Ab}	30.85 ^{Ac}	22.28 ^{Bc}	28.23 ^{Bb}	38.01 ^{Aa}	22.57 ^{Ca}	20.11 ^{Ab}	15.32 ^{Ac}	12.51 ^{Ja}	10.53 ^{Fb}	5.51 ^{Dc}	5.11 ^{Ec}	7.85 ^{Db}	10.31 ^{Ea}
	121	34.45 ^{Da}	31.52 ^{Bb}	28.85 ^{Bc}	28.51 ^{Ac}	32.31 ^{Ab}	36.21 ^{Ba}	19.91 ^{Da}	16.83 ^{Cb}	13.58 ^{Bc}	10.30 ^{Ka}	8.81 ^{Gb}	6.15 ^{Dc}	6.83 ^{Dc}	10.53 ^{Db}	15.21 ^{Ca}
SEM_{pooled} *			0.667			0.782			0.531			0.634			0.367	

Different uppercase letter superscripts indicate a significant difference among the means within a column, while lowercase superscript signifies a significant difference among the means within a raw; $p < 0.05$. * Pooled Standard Error of Mean.

4. Discussion

Adjusting the pH of the MPC suspensions before heat treatment significantly influenced the association between denatured whey proteins and casein micelles. Lowering the pH < 6.8 and heating promotes the formation of aggregates involving caseins and whey proteins. Particularly, at these lower pH levels, whey protein interactions with the

casein micelle surface during heating lead to the formation of larger particles. Moreover, the relatively high amount of whey proteins associated with micelles may have facilitated the aggregation of casein particles through cross-linking of whey proteins bound to micelles [21]. Additionally, the reduction in pH and the altered dynamic equilibrium of minerals, particularly calcium and phosphate, between the serum and MCP nanoclusters caused by heating, lead to micellar destabilization. This results in the release of κ-CN, which makes the micelles more susceptible to aggregation. This is accompanied by a reduction in α-helical structures and β-sheets, linked to the disruption of hydrogen bonds and a less orderly arrangement of protein elements [20]. The pH of a solution significantly affects the protein behaviour, particularly through its relation to the protein's isoelectric point (pI). The pI represents the pH at which a protein carries no net charge. For caseins, with a pI around 4.6, the proximity of the solution's pH to the pI influences solubility and conformational changes, affecting interactions with other molecules. This correlation between casein's pI and solution pH directly affects micellar stability and aggregation tendencies [11]. Therefore, a more acidic environment is likely to enhance electrostatic interactions among proteins by charge neutralization. This, in turn, can impact the stability of hydrogen bonds, leading to a significant reduction in both intramolecular β-sheets and α-helical content as observed in FTIR analysis (Figure 2 and Table 5) [20].

Adjusting pH to 7.5 leads to the loss and expansion of structures in the micelles, attributed to the higher surface potential with an increased susceptibility to disruption (Table 2) [22,23]. Several factors have been identified as contributing to the dissociation of casein micelles. These include a decrease in hydrophobic interactions among the caseins, increased electrostatic repulsions, and alterations in the mineral equilibrium involving calcium and phosphate [24–26]. In addition, a decrease in pH due to the liberation of hydrogen ions and changes to the dynamic equilibrium of minerals between colloidal and soluble phases with the heating considerably contributed to the destabilization of the micellar structure. Such destabilization aids in the dissociation and subsequent release of caseins into the serum, which increases the proportion of caseins in the soluble phase [27]. As a result of micellar dissociation, a reduction in particle size was observed in the MPC suspensions at pH 7.5 upon heating (Table 1) [7]. In addition, the altered charge distribution influences the stability of hydrogen bonds, manifesting a decline in β-sheets, β-turns, and α-helical structures while increasing random structures, signifying a less orderly arrangement of protein elements (Figure 2) [20].

The application of shear alters the dynamics of aggregation, shifting from perikinetic to orthokinetic aggregation. This transition involves particle collisions occurring within the flow streamlines [28]. Although the flow intensifies the frequency of collisions, it simultaneously introduces the potential for fracture of aggregates [29]. In the presence of shear, the growth dynamics and size of protein complexes are influenced by the interplay of shear-induced growth and shear-mediated breakage mechanisms [30]. In the control MPC suspensions at pH 6.8, prominent shear-induced fragmentation of protein aggregates was evident at both temperatures. This was due to an increase in all caseins and whey proteins in the non-sedimentable fraction, while their levels in the aggregated fraction were reduced (Tables 3 and 4).

At pH 6.1, shear-induced collisions between particles may enhance aggregation compared to pH 6.4 (Figure 1). While shear consistently promotes particle collisions, the pH of the environment determines their propensity to stick together, with lower pH values favouring increased aggregation. As a result, in suspensions with a pH adjusted to 6.1, a more noticeable decrease in the levels of both caseins and whey proteins in the non-sedimentable fraction was observed compared to suspensions at pH 6.4 under the combined application of heat and shear (Tables 3 and 4). On the other hand, higher electrostatic repulsion and less association of whey proteins with micelles at pH 6.4 compared to pH 6.1 may result in lower aggregation, thereby promoting prominent fragmentation (Figure 1 and Table 1). These changes in the charge distribution among proteins might have prompted conformational adjustments in proteins, resulting in a decline in intramolecular β-sheet content (Figure 2

and Table 5). The aggregation process itself could have induced alterations in protein conformation, thereby contributing to the observed reduction in β-sheet content. Protein aggregation may involve the formation of turns and loops in protein structures, elucidating the observed increase in β-turns at pH 6.1 (Table 5).

In the MPC suspensions with pH adjusted to 7.5, the application of combined heat and shear increased the casein levels in the non-sedimentable phase, with a more pronounced effect observed at 121 °C (Tables 3 and 4). The fluid drag induced by shear flow may contribute to the structural transformations into elongated shapes and the exhibition of complex behaviour. For instance, at high concentrations, casein micelles can behave like soft spheres, deforming and aligning in the direction of the flow at elevated shear rates [10]. In addition to the looser and expanded structure of the micelles, weakened hydrophobic interactions resulting from high temperatures under the influence of pH make them susceptible to destabilization affecting the overall balance between interactions. The increased collision rate with the rise in shear leads to further disruption of micelles, causing the release of more κ-CN into the non-sedimentable phase (Table 3). These shear-induced mechanical forces disrupt the secondary structure of milk proteins, particularly reducing α-helical structures, through protein deformation, unfolding, and reorientation when subjected to shear flow as observed previously [5] (Table 5).

5. Conclusions

In conclusion, the interplay between shear and pH in MPC suspensions reveals a complex dynamic of protein interactions and structural alterations. At lower pH levels, such as pH 6.1, the application of shear enhances particle collisions, leading to increased aggregation compared to those at pH 6.4. This suggests that pH influences the propensity of particles to adhere, with lower pH values favouring heightened aggregation under shear conditions. Conversely, at pH 6.4, higher electrostatic repulsion and less association of whey proteins with micelles result in less aggregation, promoting prominent fragmentation. A shear-dependent reduction in intramolecular β-sheets and α-helical content was observed at both temperatures at both pH levels suggesting alterations in protein conformation. Shear-induced fragmentation of protein aggregates was clearly noticeable in the control MPC suspensions at pH 6.8, regardless of the heating temperature. The combination of heat and shear applied to MPC suspensions at pH 6.8 resulted in a reduction in intramolecular β-sheet structures and an increase in β-turns. There was also a decrease in α-helix content and an increase in random coils. Additionally, aggregated β-sheet structures decreased compared to heated dispersions. Adjusting the pH to 7.5 yields loose and expanded micellar structures, rendering them more susceptible to shear-induced disruption. Shear, in combination with heat, intensifies collisions and disrupts micelles, leading to the release of more κ-CN into the non-sedimentable phase. At pH 7.5, the combined application of heat and shear decreased the content of α-helical elements compared to heated suspensions due to changes in protein structure.

Overall, the findings underscore the intricate relationship between shear, pH, and protein behaviour in MPC suspensions. This understanding aids dairy manufacturers in optimizing processing conditions for improved product stability, texture, and functionality. It also supports the development of processing techniques that preserve protein functionality and nutritional integrity in dairy products. Further research could delve deeper into elucidating the specific mechanisms underlying shear-induced alterations in protein structures and interactions across a broader range of pH conditions.

Author Contributions: Conceptualization, A.M.; methodology, A.M.; software, A.M. and T.V.; validation, A.M., T.V., J.C. and T.H.; formal analysis, A.M., T.V., J.C. and T.H.; investigation, A.M.; resources, A.M.; data curation, A.M.; writing—original draft preparation, A.M.; writing—review and editing, A.M., T.V., J.C. and T.H.; visualization, A.M.; supervision, T.V., J.C. and T.H.; funding acquisition, T.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Acknowledgments: The authors extend their appreciation to Victoria University for the financial and technical assistance provided.

Conflicts of Interest: Thom Huppertz is employed by the company FrieslandCampina. The research was conducted without any commercial and financial relationships with the company that could be construed as a potential conflict of interest.

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CHAPTER 5. Heat-induced changes of milk protein concentrate suspensions as affected by addition of calcium sequestering salts and shearing

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Published in *International Dairy Journal* 149, 2024, 105829

https://doi.org/10.1016/j.idairyj.2023.105829

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1. PUBLICATION DETAILS (to be completed by the candidate)

Title of Paper/Journal/Book: Heat-induced changes of milk protein concentrate suspensions as affected by addition of calcium sequestering salts and shearing

Surname: Mediwaththe First name: Anushka

Institute: Institute for Sustainable Industries and Liveab Candidate's Contribution (%): 80

Status:

Accepted and in press: ☐ Date:

Published: ☑ Date: 14/10

2. CANDIDATE DECLARATION

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Anushka Mediwaththe Digitally signed by Anushka Mediwaththe Date: 2024.02.14 09:18:57 +11'00'	14/02/24
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Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
Anushka Mediwaththe	80	Experiment design and execution, data analysis, manuscript drafting and	[redacted]	14/02/24
Jayani Chandrapala	5	Designing experiments, manuscript editing and revision		14/02/24
Thom Huppertz	5	Designing experiments, manuscript editing and revision		14/02/2024
Todor Vasiljevic	10	Designing experiments, manuscript editing, revision and journal submission		14/02/24

Updated: September 2019

International Dairy Journal 149 (2024) 105829

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Heat-induced changes of milk protein concentrate suspensions as affected by addition of calcium sequestering salts and shearing

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ARTICLE INFO

Article history:
Received 8 September 2023
Received in revised form
12 October 2023
Accepted 14 October 2023
Available online 28 October 2023

ABSTRACT

Calcium sequestering salts (CSS) such as disodium hydrogen phosphate (DSHP) and trisodium citrate (TSC) can be used to improve the heat stability and solubility of milk proteins during thermal processing. Shearing is also an inherent part of food processing that can alter protein properties and functionality. The heat stability of milk proteins was investigated under combined temperatures (90 °C/5 min; 121 °C/2.6 min) and shear (100, 1000, 1500 s^{-1}) with 10, 20, or 30 mM DSHP or TSC. The calcium-binding capacity of individual CSS predominantly determined the impact of shear. With DSHP, the effect of shear was more pronounced at high temperatures and shear rates than with TSC. The significant influence of shear on casein micelles in DSHP-containing dispersions suggested that DSHP's lower calcium binding affinity minimised micellar disruption and led to a pronounced shear impact. Shear combined with heat considerably impacts the milk system in the presence of CSS.

1. Introduction

Milk protein concentrates (MPCs) serve as an essential protein source in protein-enriched beverages due to their high protein content (50–85 %) and casein-to-whey protein ratio similar to milk (Agarwal, Beausire, Patel, & Patel, 2015). However, the heat stability of MPC powders poses a significant challenge as they are subjected to rigorous heat treatments, such as ultra-high temperature (UHT) or retorting, to extend their shelf life. Several factors, including processing conditions, composition, physical state, protein concentration, ionic composition, storage time, and temperature, directly influence the chemical and physical stability of MPC proteins (Corredig, Nair, Li, Esphari & Zhao, 2019; Singh, 2004). A higher protein content can result in increased calcium activity and reduced heat stability during evaporation and drying, leading to lower solubility in powdered MPC products (Huppertz, Fox, & Kelly, 2018).

The loss of solubility is attributed to interactions between caseins, predominantly α_S- and β-casein, on the surface of the powder particles. These interactions impede water transfer during hydration, cause the migration of residual fat to the particle surface, and slow the release of casein micelles from the dispersed powder (Havea, 2006). Furthermore, the composition of undissolved material in MPC varies significantly (Udabage, Puvanenthiran, Yoo, Versteeg, & Augustin, 2012). Interactions between caseins and minor whey proteins appear to be one of the reasons leading to insolubility (Havea, 2006). Increasing reconstitution time and temperature generally improves solubility, as proteins lose their secondary and tertiary structures due to the destabilisation of non-covalent interactions, typically at temperatures ~50 °C (Fang, Selomulya, Ainsworth, Palmer, & Chen, 2011). However, higher temperatures used during further processing, such as UHT treatment and sterilisation, lead to decreased solubility as proteins aggregate and precipitate due to inter-protein hydrophobic and covalent interactions, resulting in physical instability, phase separation, aggregation, precipitate formation, and gelation (Carr & Golding, 2016).

To prevent the development of insolubility in MPC, methods such as the addition of calcium sequestering salts (CSS), treatment with cation exchange resins, and low-pH membrane filtration are employed. These techniques reduce the colloidal calcium phosphate (CCP) content and increase non-micellar casein content, thereby enhancing heat stability (Carr & Golding, 2016). These CSS bind to polyvalent metal ions resulting in indirect demineralisation of casein micelles. However, excessive concentrations of CSS can

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https://doi.org/10.1016/j.idairyj.2023.105829

decrease heat stability by sequestering critical levels of CCP from the micelles, leading to micellar integrity loss (Broyard & Gaucheron, 2015; Lucey & Horne, 2009; Mizuno & Lucey, 2005). Typically, phosphate and citrate concentrations up to 40 mmol per kg skim milk solids are commonly used as heat stabilisers in the dairy industry (Augustin & Clarke, 1990).

In addition to environmental conditions, mechanical forces, such as shear, can alter the properties of casein micelles, resulting in different physical properties within the system (Ranadheera et al., 2019). Shear forces are imposed during various processing steps, including pumping, stirring, mixing, homogenising, and flow-through heat exchangers. These forces directly impact the stability of proteins and the mineral environment in the milk system (Mediwaththe, Bogahawaththa, Grewal, Chandrapala, & Vasiljevic, 2018a; Mediwaththe, Chandrapala, & Vasiljevic, 2018b). Significant velocity gradients generated by shear forces lead to conformational transitions, unfolding, and exposure of hydrophobic amino acids, which induce interactions among and within protein structures and promote protein aggregation (Di Stasio & De Cristofaro, 2010). Joined effects of temperature and shear forces would impose greater structural changes thus leading to more pronounced denaturation and aggregation. Ranadheera et al. (2019) reported that aggregates of different compositions were formed at different pH and temperatures with varying applied shear. The effect was amplified at low pH (4.6 and 2.0) and high shear (1000 s^{-1}) under which conditions created aggregates were partially dissociated by shear. This demonstrates the application of shear has a potential of modulating structural properties of solubilised MPC dispersions in conjunction with other environmental factors.

While sterilising at high temperatures after the addition of CSS is a common strategy to improve heat stability and solubility of MPCs, the simultaneous application of shear has not been thoroughly investigated. Thus, the primary objective of this study was to explore the impact of varying levels of CSS (phosphate or citrate) on the protein stability of high-protein milk dispersions containing 8% (w/w) proteins, a commonly used concentration in dairy beverage manufacturing. These systems were subjected to different heat treatments (90 °C/5min and 121 °C/2.6 min) and shear rates (100, 1000, or 1500 s^{-1}). Understanding the interplay between these processing conditions and the physical and chemical changes involving milk proteins is crucial for enhancing MPC functionality, allowing the industry to tailor MPC functionality by manipulating processing parameters.

2. Materials and methods

2.1. Materials

MPC powder was obtained from Bega Cheese (Bega, NSW, Australia) and stored in air-tight plastic containers at −20 °C. The composition of the MPC powder was 78.7% (w/w) protein, 1.3% (w/w) fat, 4.7% (w/w) lactose and 7.5% (w/w) ash as per manufacturer's declaration. All the chemicals used for analysis were obtained from Sigma–Aldrich Pty Ltd (Castle Hill, NSW, Australia) and ultrapure water (Milli-Q water, Merck Millipore, Bayswater, Vic, Australia) was used at all times.

2.2. Sample preparation and treatment

The MPC powder was reconstituted in Milli-Q water to obtain dispersions containing ~8% protein (w/w). The dispersion was continuously stirred for 1 h at 50 °C for complete solubilisation of powder and kept at 4 °C overnight for further hydration (Liyanaarachchi & Vasiljevic, 2018). The following day, the samples were equilibrated at 25 °C for 1 h before the start of experiments. A CSS, either phosphate (200 mM di-sodium hydrogen phosphate solution) or citrate (200 mM tri-sodium citrate solution), was prepared and gradually introduced into MPC dispersions. The protein content of the MPC dispersions was initially adjusted to achieve a final concentration of 8% protein (w/w) after the CSS was added. This process aimed to obtain final CSS concentrations of 10, 20, or 30 mM. The addition was carried out under constant stirring at 25 °C, followed by allowing the mixtures to equilibrate for a duration of 60 min. These dispersions were processed in a rheometer immediately after the final pH of the milk was adjusted to 6.8 by a dropwise addition using 0.1 M HCl, accounting for the change in the volume resulting in the final concentration (Ramchandran, Luo, & Vasiljevic, 2017; Renhe, Indris, & Corredig, 2018).

Prepared MPC dispersions were heated at two temperature/time combinations (90 °C for 5 min and 121 °C for 2.6 min) at a shear rate of 100, 1000 or 1500 s^{-1} in a pressure cell (CC25/PR-150) of a rheometer (Physica MCR 301 series, Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with a constant pressure of 250 kPa following the method of Liyanaarachchi, Ramchandran, & Vasiljevic (2015). Samples subjected to heating under two temperatures were heated at a rate of 5 °C min^{-1} to the required temperature and held there for the required time and cooled at a rate of 5 °C min^{-1}. The pH of each treated sample was measured immediately after treatment using a pH meter (WTW Inolab pH 720, Weilheim, Germany) and the first stable endpoint was recorded. The pH of each suspension was initially standardised, and final pH measurements were taken after each treatment. No substantial change of pH was observed following each treatment. A portion of the treated sample was subsequently ultracentrifuged (Beckman Optima L-70 Ultracentrifuge, Indianapolis, IN, USA) at 100,000×g for 1 h at 20 °C to obtain the serum phase of the dispersions. The supernatant was carefully removed and used for further analysis.

2.3. Particle size and zeta potential measurements

Particle size and zeta potential measurements were performed immediately after treatments using a Zetasizer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) as described by Liyanaarachchi et al. (2015). Treated samples were diluted 1000 times using UF permeate, obtained by ultrafiltration of skim milk at 15 °C with a SEPA CF membrane module and polyethersulfone (PES) membrane (190 × 140 mm) with a molecular cut-off of 10 kDa, acquired from Sterlitech Corporation (Kent, WA, USA). Refractive index for the casein micelle in the MPC and the dispersant were set at 1.57 and 1.34, respectively (Griffin & Griffin, 1985).

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Following the treatments, all the treated and control samples and supernatants of centrifuged samples were mixed with sodium dodecyl sulphate (SDS) sample buffer at 1:25 (v/v) ratio. Both non-reducing and reducing (with β-mercaptoethanol as the reducing agent) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously (Bogahawaththa, Buckow, Chandrapala, & Vasiljevic, 2018). The gel images were captured by Image Lab 5.1 software (Bio-Rad Laboratories, Galesville, NSW, Australia). The intensity of reducing gel proteins in the supernatants was expressed as a percentage of their corresponding proteins in control bulk.

2.5. Calcium determination using inductively coupled plasma emission spectrometry

For the determination of non-sedimentable calcium, samples were prepared by dissolving ash obtained after combustion in

muffle furnace at 550 °C in 10 mL of 1M HNO_3 acid and water to acquire 0.1% TS content. Five standard solutions containing concentrations of Ca from 0.02 to 1% (w/w) were prepared as described by Chandrapala et al. (2015). Non-sedimentable calcium content of standards and supernatants after each treatment were analysed using inductively coupled plasma (ICP) atomic emission spectrometer (ICP E Multitype, Shimadzu corporation, Kyoto, Japan) according to the method of Martinie & Schilt (1976). The wavelength of 318 nm was used for quantification of Ca.

2.6. Ionic calcium concentration measurements

The ionic calcium in the serum phase was measured within 2 h after each treatment using a Calcium Ion Selective electrode connected to a pH meter (InoLab, WTW GmbH, Ingolstadt, Germany). Calcium chloride was used for the preparation of a standard curve and the ionic strength was adjusted by the addition of KCL to the standards (Daniloski, McCarthy, Vasiljevic, 2022; Markoska, Huppertz, Grewal, & Vasiljevic, 2019).

2.7. Statistical analysis

Each experiment was conducted in triplicate for each MPC suspension and statistical tests were performed with IBM SPSS statistics software (version 28.0.1.0, IBM Corp., Armonk, NY) using a general linear model (GLM) protocol with CSS, temperature/time combination and shearing as the main factors The level of significance was set at $P \leq 0.05$.

3. Results

3.1. Calcium distribution of milk protein concentrate upon addition of CSS

The control MPC dispersion at 25 °C contained ~6 mM of non-sedimentable calcium, out of which ~3 mM was in the form of ionic calcium in the serum phase (Table 1). As expected, the addition of CSS reduced the content of non-sedimentable and ionic calcium through different mechanisms: phosphate addition precipitates calcium, whereas citrate dissolves colloidal calcium (Tessier & Rose, 1958). For this reason, addition of DSHP up to 10 mM concentration resulted in a significant initial decline in ionic calcium (~1.3 mM), which level remained fairly constant upon further addition (Table 1). Upon the addition of 10 mM DSHP, the non-sedimentable calcium levels decreased to ~2.7 mM, which had initially been associated with either citrate or phosphate or linked with non-sedimentable casein (Bijl, Huppertz, van Valenberg, & Holt, 2019) (Table 1). As opposed to ionic calcium, further addition of DHSP returned the concentration of non-sedimentable calcium back to its initial level (Table 1). This likely occurred due to the disruption of casein micelles resulting from their interaction with structural elements within the micelles (e.g., peptisation), or possibly due to the partial solubilisation of colloidal calcium from the casein micelles to a lesser extent (Garcia, Alting, & Huppertz, 2023). On the other hand, the addition of TSC at 25 °C resulted in a more gradual decline of ionic calcium, reaching a similar level to that of DHSP addition at 30 mM (Table 1). At the same time, the concentration of the non-sedimentable calcium gradually but significantly increased reaching 40.6 mM upon 30 mM addition of TSC, indicative of solubilisation of colloidal calcium (Tessier & Rose, 1958) (Table 1).

Heating the MPC dispersion without added CSS at 90 or 121 °C also reduced the levels of ionic and non-sedimentable calcium, although not to the levels achieved by sequestering (Table 1). In the presence of CSS, impact of heat on the levels of ionic calcium was augmented as it further declined compared with the corresponding controls. However, no difference in the levels of ionic calcium was observed between the applied heat treatments. Interestingly, heating of dispersions containing 10 mM DSHP did not have a major effect on the non-sedimentable calcium, which remained at similar levels; however, this changed when the dispersion containing either 20 or 30 mM of DSHP was heated at 121 °C, which resulted in increased non-sedimentable calcium. The maximum of the non-sedimentable calcium achieved by addition of 30 mM DHSP

Table 1
Non-sedimentable and ionic calcium in supernatants of 8% MPC dispersions upon addition of disodium hydrogen phosphate (DSHP) or trisodium citrate (TSC) at different concentrations subjected to heating at 90 or 120 °C and shearing at 100, 1000 or 1500 s^{-1}.[a]

Calcium sequestering salt	Temp. (°C)	Calcium sequestering salt conc. (mM)	Ionic calcium (mM)				Non-sedimentable calcium (mM)			
			Shear rate (s^{-1})				Shear rate (s^{-1})			
			0	100	1000	1500	0	100	1000	1500
None (control)	25	0	3.06^{A}				6.2^{GHI}			
	90	0	2.79^{Ba}	2.65^{Ab}	2.70^{Ab}	2.81^{Aa}	4.1^{JKb}	3.7^{EFb}	5.5^{Ga}	6.1^{FGa}
	121	0	2.69^{Bc}	2.80^{Ab}	2.82^{Aab}	2.87^{Aa}	2.7^{Lb}	2.8^{EFb}	3.28^{Hb}	4.3^{Ha}
DSHP	25	10	1.32^{FGHI}				2.7^{L}			
		20	1.34^{FGHI}				4.5^{J}			
		30	1.36^{FGH}				6.5^{GH}			
	90	10	1.30^{FGHIab}	1.35^{DEa}	1.31^{CDEFab}	1.22^{DEb}	3.0^{KLa}	3.2^{EFa}	2.1^{Ib}	3.2^{Ia}
		20	1.28^{GHIa}	1.26^{DEFGa}	1.22^{EFGa}	1.18^{Ea}	2.5^{Lb}	9.3^{CDEFa}	9.1^{Ea}	8.6^{Ea}
		30	1.19^{Ia}	1.29^{DEFa}	1.17^{FGa}	1.18^{Ea}	2.5^{La}	5.8^{EFb}	7.1^{Fb}	8.6^{Eb}
	121	10	1.61^{DEa}	1.67^{BCa}	1.18^{FGb}	1.20^{DEb}	2.1^{Lb}	2.7^{Fa}	3.8^{Ha}	3.0^{Ia}
		20	1.32^{FGHIa}	1.33^{DEa}	1.26^{DEFa}	1.18^{Ea}	5.3^{HIJc}	7.7^{DEFa}	7.1^{Fb}	7.8^{Ea}
		30	1.21^{HIa}	1.12^{Gab}	1.07^{Gab}	1.02^{Fb}	5.1^{IJab}	4.7^{EFb}	5.3^{Ga}	5.0^{GHab}
TSC	25	10	1.70^{CD}				6.9^{G}			
		20	1.45^{EF}				27.8^{B}			
		30	1.31^{FGHI}				34.6^{A}			
	90	10	1.78^{Ca}	1.76^{Ba}	1.62^{Bb}	1.48^{Bc}	17.2^{Da}	14.8^{CDb}	14.2^{Cb}	14.9^{Db}
		20	1.42^{FGa}	1.24^{EFGb}	1.37^{CDEa}	1.35^{Ca}	24.1^{Cab}	25.1^{ABa}	22.2^{Bb}	23.0^{Abc}
		30	1.38^{FGa}	1.34^{DEa}	1.38^{CDa}	1.36^{BCa}	28.7^{Ba}	31.1^{Aa}	28.9^{Aa}	20.4^{Bb}
	121	10	1.76^{CDa}	1.54^{Cb}	1.47^{BCb}	1.19^{Ec}	10.0^{Fa}	10.1^{CDEa}	9.1^{Eb}	6.3^{Fc}
		20	1.35^{FGHIab}	1.39^{Da}	1.29^{DEFb}	1.32^{CDab}	15.3^{Eb}	17.3^{BCa}	12.2^{Dc}	14.8^{Db}
		30	1.26^{GHIa}	1.16^{FGa}	1.26^{DEFa}	1.17^{Ea}	24.3^{Cb}	29.4^{Aa}	21.7^{Bc}	18.9^{Cd}
Standard error of mean			0.04				0.35			

[a] Means in a column with different superscript uppercase letters and a row with different superscript lowercase letters differ significantly (p <0.05).

(~5.1 mM) was still substantially lower than that of the control (~6.5 mM) (Table 1). In dispersions where TSC was added, the increase in heat load and CSS level caused a gradual decrease in the ionic calcium content, while the non-sedimentable calcium levels gradually increased, showing a higher increase than in the DSHP added dispersions. The non-sedimentable calcium levels were significantly higher than those of the control, indicating a greater solubilisation of colloidal calcium. However, upon heat treatment, there was a gradual decline in non-sedimentable calcium levels at each TSC concentration probably due to the formation of less non-sedimentable calcium citrate complexes (Table 1).

The combined application of heat and shear initially led to a decrease in the levels of both ionic and non-sedimentable calcium at a shear rate of 100 s^{-1} when compared with the control at 25 °C. This decrease was observed at both 90 °C and 121 °C. These levels gradually increased, reaching their peak at a shear rate of 1500 s^{-1}, and ultimately reaching similar levels to those of the control at 25 °C (Table 1). However, in DSHP-added dispersions, there was not much difference observed in the levels of ionic and non-sedimentable calcium with the application of combined heat and shear. Similarly, the levels of ionic calcium did not change upon the application of combined heat and shear in the TSC-added dispersions. However, the applied shear resulted in a gradual decline of non-sedimentable calcium levels at both 90 °C and 121 °C, at all CSS levels, with a more pronounced effect at 121 °C (Table 1).

3.2. Particle size distribution and zeta potential of milk protein concentrate upon addition of CSS

The control dispersion had an average particle size of ~208 nm, which decreased upon addition of DSHP (~190 nm), likely indicating some disruption of casein micelles, which was not concentration-dependant (Table 2; Supplementary material Figs. S1 and S2). On the other hand, the addition of TSC resulted in an increase in average particle size to ~224 nm in a partially concentration-dependant manner, suggesting some swelling of the micelles (Table 2; Supplementary material Figs. S1 and S2).

The application of heat to the control MPC dispersions resulted in a gradual decrease in particle size with increasing temperature (~176 nm at 121 °C) (Table 2; Supplementary material Fig. S1). The size of casein micelles in a milk suspension depends on the initial pH and the duration of heat treatment (Anema, 2023). Specifically, at a pH of 6.8, the application of heat results in the dissociation of κ-CN, α_S-CN, and β-CN, as confirmed by observed data from SDS-PAGE analysis. This dissociation was linked to a significant reduction in particle size. Additionally, it was evident that low levels of denatured whey proteins were associated with the casein micelles, as the content of both β-LG and α-LA declined in the serum phase during heating, although the κ-CN content increased. Consequently, it is possible that the decrease in particle size could be attributed to either the dissociation of casein micelles or the formation of smaller whey protein aggregates. Similar observations were made by Anema (2023). Moreover, it is likely that the substantial loss of charged κ-CN from the casein micelles, along with the denatured whey proteins associated with it, could contribute to the dehydration and subsequent shrinkage of the remaining casein micelles. This, in turn, leads to a reduction in particle size (Anema, Lee, & Klostermeyer, 2022). Heating the dispersions with added DSHP further decreased the average particle size compared with the corresponding controls, indicating further solubilisation of the colloidal calcium. Similarly, in dispersions with added TSC, the application of heat in combination with the levels of TSC resulted in a reduction in the average particle size. However, this trend changed when the dispersion containing 30 mM TSC was heated at 121 °C, resulting in a significant increase in particle size (~377 nm), indicating aggregation of casein micelles (Table 2; Supplementary material Figs. S1 and S2).

The combined application of heat and shear to the control dispersions resulted in a reduction in particle size (~177 nm at 121 °C) (Table 2; Supplementary material Fig. S1). However, no significant difference was observed in the average particle size between the different levels of applied shear (Table 2; Supplementary material Fig. S1). Likewise, the combined application of heat and shear to MPC dispersions containing added

Table 2
Average particle size and zeta potential of 8% MPC dispersions upon addition of disodium hydrogen phosphate (DSHP) or trisodium citrate (TSC) at different concentrations subjected to heating at 90 or 120 °C and shearing at 100, 1000 or 1500 s^{-1}.[a]

Calcium sequestering salt	Temp. (°C)	Calcium sequestering salt conc. (mM) (mM)	Particle size (nm)				Zeta potential (mV)			
			Shear rate (s^{-1})				Shear rate (s^{-1})			
			0	100	1000	1500	0	100	1000	1500
None (control)	25	0	208^{CD}				-23.7^{ABC}			
	90	0	181^{FGa}	180^{Ca}	180^{CDa}	177^{CDa}	-21.5^{Aa}	-24.3^{Bb}	-21.5^{Aa}	-21.3^{Aa}
	121	0	176^{GHa}	177^{CDEa}	175^{CDEa}	177^{CDa}	-21.3^{Ab}	-20.8^{Aab}	-19.1^{Aa}	-21.7^{Ab}
DSHP	25	10	196^{E}				-22.6^{AB}			
		20	192^{EF}				-24.8^{BCD}			
		30	190^{EF}				-25.1^{BCDE}			
	90	10	166^{HIa}	170^{EFa}	172^{CDEa}	170^{DEa}	-29.6^{FGHIb}	-26.1^{BCa}	-26.0^{Ba}	-26.3^{Ba}
		20	161^{Ia}	149^{Gb}	162^{Ea}	162^{EFa}	-30.5^{HIa}	-30.3^{DEa}	-26.6^{Ba}	-27.4^{Ba}
		30	148^{Gb}	153^{Ga}	144^{Fb}	153^{FGa}	-27.8^{DEFGHa}	-27.5^{CDa}	-28.1^{BCDa}	-30.4^{CDEb}
	121	10	168^{GHIa}	171^{EFa}	168^{DEa}	168^{DEa}	-26.7^{CDEFa}	-27.6^{CDa}	-26.6^{Ba}	-27.2^{Ba}
		20	158^{IJa}	148^{Gb}	147^{Fb}	151^{Gb}	-28.9^{FGHIa}	-30.6^{Ea}	-29.8^{DEFa}	-29.4^{BCDa}
		30	189^{EFa}	180^{CDa}	182^{Ca}	180^{Ca}	-34.0^{Ia}	-34.9^{Fa}	-33.9^{Ga}	-32.1^{DEa}
TSC	25	10	198^{DE}				-25.2^{BCDE}			
		20	224^{B}				-27.3^{DEFG}			
		30	224^{B}				-28.9^{FGHI}			
	90	10	171^{GHIa}	171^{EFa}	171^{CDEa}	160^{EFGb}	-28.0^{EFGHa}	-28.8^{CDEa}	-29.3^{CDEa}	-30.1^{CDEa}
		20	166^{HIa}	153^{Gb}	171^{Ea}	162^{EFa}	-31.6^{IJab}	-31.0^{Eab}	-29.9^{DEFa}	-32.7^{Eb}
		30	211^{Cab}	205^{Bb}	219^{Ba}	201^{Bb}	-31.7^{IJa}	-31.0^{Ea}	-31.8^{FGa}	-30.6^{CDEa}
	121	10	160^{IJb}	168^{Fab}	166^{DEab}	169^{DEa}	-29.7^{GHIb}	-29.9^{DEb}	-27.2^{BCa}	-28.7^{BCab}
		20	175^{GHb}	165^{Fc}	184^{Ca}	185^{Ca}	-29.9^{GHIa}	-31.2^{Ea}	-31.27^{EFa}	-31.5^{CDEa}
		30	377^{Ac}	369^{Ac}	399^{Ab}	476^{Aa}	-26.9^{DEFGa}	-28.8^{CDEb}	-28.4^{BCDb}	-28.7^{BCb}
SEM*			3.23				0.80			

[a] Means in a column with different superscript uppercase letters and a row with different superscript lowercase letters differ significantly (p <0.05).

DSHP resulted in smaller particles (~160–180 nm) in general, compared with the corresponding dispersions with similar DSHP concentrations at 25 °C, without a significant effect of the shear level (Supplementary material Fig. S2A–C). The particle size distribution of TSC-added dispersions followed a similar trend under combined heat and shear at 90 °C as the DSHP-added dispersions. At 90 °C, smaller particles (~160–175 nm) were observed in the TSC-added dispersions with no significant impact of the applied shear. However, at 121 °C, the average particle size significantly increased with applied shear, reaching up to ~476 nm at 1500 s^{-1} and at 30 mM TSC (Supplementary material Fig. S2C–E). As observed in the particle size distribution data at 30 mM, a small peak in the range of 30–80 nm at 100 s^{-1} likely indicates dissociated casein particles. At 1500 s^{-1}, peaks in the range of 3000–8000 nm would indicate shear-induced aggregation (Supplementary material Fig. S2F).

Zeta potential of the control at 25 °C was ~−24 mV, and the addition of DSHP did not change it substantially (Table 2). On the other hand, the addition of TSC at 25 °C resulted in a gradual but significant increase in a negative zeta potential concomitant with a rise in concentration, reaching −29 mV at 30 mM likely due to higher calcium-binding capacity compared with that of DSHP (de Kort, 2012) (Table 2). Heating the control samples did not result in any significant difference in the zeta potential at either temperature. As expected, heating the dispersions containing CSS resulted in a higher net negative zeta potential in MPC dispersions due to a considerable reduction in ionic calcium (Tsioulpas, Koliandris, Grandison, & Lewis, 2010). The net-negative zeta potential in TSC added dispersions appeared to be higher since the calcium binding capacity of TSC is comparatively greater than that of DSHP. However, no apparent trend was observed with the heating load and level of CSS addition (Table 2).

Combined application of heat and shear did not affect the zeta potential in the control MPC dispersions as indicated in Table 2. On the other hand, the zeta potential of the dispersions with added CSS appeared to be more negative upon shearing during heating compared with that of the control dispersions. Nevertheless, no clear correlation could be established between the magnitude of applied shear and zeta potential (Table 2).

3.3. Partitioning of proteins upon addition of CSS at different temperature and shear rates

All protein aggregates present in the non-reducing gels of both unheated and heated controls, as well as dispersions with added CSS, disappeared in reducing gels, indicating that they were formed by thiol/disulphide interactions (Supplementary material Figs. S3–7). The protein profiles obtained under reducing conditions from these supernatants indicated that major whey proteins, including β-lactoglobulin and α-lactalbumin, were present in similar quantities regardless of the treatment (Supplementary material Figs. S3–7). A general observation of the non-reducing gels suggested a noticeable effect of each treatment on the extent of aggregation (Supplementary material Figs. S3–7).

The addition of CSS at 25 °C resulted in an increase in α_S-, β-, and κ-CNs in the supernatant due to the dissociation of casein micelles. This effect was more pronounced in the presence of TSC due to its higher calcium binding capacity, which resulted in greater micellar dissociation (Table 3; Supplementary material Figs. S3–7). Heating of the control samples resulted in a gradual decrease in the concentration of β-LG and α-LA in the supernatant parallel to the rise in temperature due to heat-induced aggregation (Tables 3 and 4; Supplementary material Fig. S3). On the other hand, a corresponding increase was observed for α_S-, β-, and κ-CNs, with a relatively greater rise in κ-CN concentration compared with that of

Table 3
Intensity of caseins in supernatants as a % of their intensity in the control bulk dispersions subjected to different treatments resolved under reducing electrophoretic conditions and quantified using a ChemiDoc imager.

Temp. (°C)	Shear (s^{-1})	0 mM			10 mM			20 mM			30 mM		
		α_S-CN	β-CN	κ-CN	α_S-CN	β-CN	κ-CN	α_S-CN	β-CN	κ-CN	α_S-CN	β-CN	κ-CN
Control													
25	0	3.5	5.3	23.2									
90	0	3.8	6.1	24.5									
	100	3.0	5.6	30.6									
	1000	2.8	5.3	32.3									
	1500	2.0	2.6	33.1									
121	0	8.5	8.6	32.9									
	100	7.5	8.0	31.7									
	1000	6.8	5.8	33.5									
	1500	6.0	4.9	35.1									
Disodium hydrogen phosphate													
25	0				13.7	10.8	24.8	21.8	21.3	27.1	17.3	28.2	30.2
90	0				14.1	12.7	39.3	17.3	30.7	42.1	31.1	58.4	63.2
	100				15.3	13.1	40.8	16.8	34.7	43.7	41.7	57.2	65.0
	1000				18.0	14.5	55.1	18.5	47.3	72.3	58.5	63.0	72.5
	1500				19.8	15.3	63.1	20.2	49.9	73.5	60.4	64.5	76.8
121	0				21.6	18.5	58.8	20.5	42.5	62.1	45.4	65.3	75.7
	100				27.8	17.3	59.2	19.9	41.8	62.7	49.5	66.7	81.8
	1000				29.4	19.3	60.3	63.5	88.3	82.3	60.8	78.2	87.8
	1500				30.5	23.1	62.5	55.1	81.6	88.7	72.0	88.1	91.3
Trisodium citrate													
25	0				18.5	23.5	51.8	33.0	35.8	68.1	58.1	68.2	72.3
90	0				23.5	31.2	65.3	38.3	45.3	75.7	63.8	73.2	80.8
	100				24.1	32.5	61.3	37.9	46.1	72.1	64.5	75.3	79.7
	1000				25.3	35.8	55.4	35.3	42.5	68.1	61.8	68.8	71.5
	1500				17.8	27.8	58.1	38.7	38.1	61.3	55.3	60.3	78.8
121	0				28.5	33.3	72.3	48.3	55.5	83.1	68.8	78.2	86.5
	100				27.3	28.5	75.8	49.1	53.1	82.9	67.9	75.3	88.5
	1000				25.5	26.4	68.3	38.2	47.8	77.5	62.3	62.1	79.1
	1500				23.1	23.1	71.2	40.8	43.6	73.5	58.5	58.3	74.4

Table 4
Intensity of whey proteins in supernatants as a % of their intensity in the control bulk dispersions subjected to different treatments resolved under reducing electrophoretic conditions and quantified using a Chemidoc imager.

Temp. (°C)	Shear (s^{-1})	0 mM		10 mM		20 mM		30 mM	
		β-LG	α-LA	β-LG	α-LA	β-LG	α-LA	β-LG	α-LA
Control									
25	0	98.8	99.3						
90	0	43.5	57.8						
	100	46.0	58.3						
	1000	48.3	63.8						
	1500	53.1	65.2						
121	0	33.5	35.9						
	100	34.3	34.7						
	1000	38.1	36.8						
	1500	47.5	38.4						
Disodium hydrogen phosphate									
25	0			94.5	95.1	86.5	89.2	88.0	87.1
90	0			62.1	78.9	56.8	59.8	58.3	59.8
	100			69.5	76.4	63.8	58.1	66.4	57.6
	1000			63.5	72.9	51.2	50.6	53.7	53.0
	1500			59.8	71.9	46.8	49.3	51.4	51.8
121	0			48.4	54.6	43.7	52.3	44.1	53.6
	100			47.8	53.4	43.4	53.9	42.8	52.5
	1000			48.1	53.9	41.1	52.5	41.9	52.3
	1500			46.5	52.5	42.1	51.9	42.3	51.8
Trisodium citrate									
25	0			90.7	89.4	93.8	80.2	82.5	80.3
90	0			56.2	76.7	51.4	61.5	51.0	57.8
	100			52.1	69.8	55.6	64.0	49.6	52.9
	1000			51.4	61.4	46.3	53.4	45.5	54.3
	1500			49.8	57.3	46.3	54.4	46.1	53.0
121	0			43.4	55.9	42.1	52.2	41.2	52.1
	100			41.5	54.3	41.3	53.0	41.8	51.6
	1000			41.0	54.1	41.0	52.8	40.9	52.0
	1500			40.9	54.3	40.9	52.2	40.8	51.3

other caseins, suggesting its further dissociation from casein micelles. The heat load, in combination with CSS level, increased the free caseins in the supernatant while further reducing β-LG and α-LA. The heat-induced unfolding of whey proteins enhanced the further aggregation of proteins, reducing serum whey protein levels (Tables 3 and 4; Supplementary material Figs. S4 and S6). This effect was more pronounced in TSC-added dispersions due to the stronger sequestering capacity.

The application of heat and shear to the control samples resulted in a higher concentration of β-LG, α-LA, and κ-CN in the supernatant at both 90 °C and 121 °C compared with heated controls, with a greater increase observed at 90 °C, indicating that the shear forces caused fragmentation of the aggregates. The increase in β-LG, α-LA, and κ-CN was prominent at high shear rates of 1000 s^{-1} and 1500 s^{-1} (Table 3; Supplementary material Fig. S3). However, the heat load in combination with shear released less α_S- and β-CNs into the serum phase at both temperatures. Similar to the heated MPC dispersions with added DSHP, the combined application of heat and shear to DSHP-added dispersions resulted in a marked decrease in β-LG in the supernatant, especially at higher concentrations, while comparatively less reduction in α-LA was observed, with a more pronounced effect at 121 °C. A greater increase in free α_S-CN, β-CN, and κ-CN was noticed in the supernatant at 121 °C at both 20 mM and 30 mM concentrations, reaching their greatest concentrations at 1000 and 1500 s^{-1} shear rates, stipulating significant shear-induced dissociation of casein micelles (Supplementary material Fig. S4). In TSC-added dispersions, combined heat and shear resulted in much higher reduction in whey proteins compared with DSHP added dispersions. Shear-dependent gradual decline of whey proteins was prominent at 90 °C, reaching the lowest at 30 mM and at 1500 s^{-1}. In addition, all the α_S-, β- and κ-CN levels were also declined in the serum affected by shear. When compared with MPC dispersions with added DSHP, those with added TSC showed relatively higher levels of α_S-, β-, and κ-CN in the supernatant with the combined application of heat and shear at all three concentrations, indicating comparatively higher micellar dissociation (Table 3; Supplementary material Figs. S6 and S7).

4. Discussion

The results indicate that the behaviour of proteins in the control MPC dispersions was affected by the combined application of heating and shearing. SDS-PAGE analysis revealed that the impact of shear on untreated controls was more pronounced at 90 °C, leading to an increase in β-LG, α-LA, and κ-CN in the serum. This increase is likely due to prominent shear-induced disruption of protein aggregates (Bogahawaththa & Vasiljevic, 2020; Mediwaththe et al., 2018a). Under shear, the net growth rate and size of protein complexes are determined by the balance between shear-induced growth and shear-controlled breakage (Steventon, Donald, & Gladden, 1994). At 121 °C, shear-induced fragmentation was observed to a lesser extent compared with 90 °C, indicating a relatively enhanced shear-induced aggregation through hydrophobic interactions, as well as thiol-disulphide interactions, to a certain extent.

CSS can have different modes of action in the disruption of casein micelles (Vujicic, DeMan, & Woodrow, 1968). All of them can complex with free ionic calcium in solution, leading to a reduction of non-sedimentable ionic calcium levels, as observed in both DSHP and TSC added MPC dispersions at 25 °C. The impact of CSS was further enhanced by increasing temperature and shear, resulting in a greater reduction of ionic calcium from the non-sedimentable phase. CSS can become more reactive upon heating and shearing due to several reasons. Higher temperatures provide more energy to the system, increasing collision frequency and enhancing molecular mobility, thereby promoting CSS-protein interactions (Mejares, Chandrapala, & Huppertz, 2023). Similarly, shear forces facilitate contact between CSS and proteins. Shear-induced disruption of casein micelles exposes more binding sites for CSS, promoting their penetration into the protein matrix and increasing reactivity (Mediwaththe et al., 2018a). Furthermore, shear-induced changes in protein conformation and aggregation state expose reactive sites and release metal ions from protein-binding sites, which can be readily sequestered by the added CSS, further increasing their reactivity (Rajan et al., 2021). The combined effect of heating and shearing enhances the reactivity of CSS, allowing them to interact more effectively with proteins and alter the stability and structure of the system. However, it is important to note that the applied heat and shear to DSHP-treated dispersions did not result in observable effects on both non-sedimentable and ionic calcium levels (Table 2).

In TSC-treated dispersions, the combined effect of heat and shear led to a significant reduction in non-sedimentable calcium levels, potentially due to the formation of calcium citrate complexes cooperating with the colloidal phase (de Kort, 2012). However, changes in serum calcium levels alone may not necessarily indicate micellar disruption. Other factors, such as structural and compositional changes in milk proteins, can provide valuable insights into the underlying mechanisms and potential micellar disruption (de Kort, Minor, Snoeren, Van Hooijdonk, & Van Der Linden, 2009).

Serum protein levels appeared to be not affected in DSHP added dispersions at 25 °C suggesting minimal impact on the protein-mineral balance (Table 3; Supplementary material Figs. S4 and S5). However, an increase in temperature and DSHP concentration led to a notable rise in non-sedimentable caseins (Table 3;

Supplementary material Figs. S4 and S5). In phosphate-based CSS with lower concentrations, the introduction of additional calcium phosphate does not displace the calcium phosphate within the micelles. However, when a higher level of phosphate is added, it results in the displacement of calcium from the micellar phase, leading to the demineralisation and subsequent solubilisation of the casein (Gaucher, Piot, Beaucher & Gaucheron, 2007). The orthophosphate anion in DSHP is believed to interact directly with positively charged amino acid residues and indirectly through calcium bridges with casein micelles. These interactions result in the formation of insoluble complexes between caseinate and calcium phosphate (Mizuno & Lucey, 2007). The alteration in the equilibrium between proteins and minerals, arising from the sequestration of calcium, results in the dissolution of colloidal calcium phosphate. This dissolution, in turn, results in the release of specific casein proteins from the micelles, or alternatively, leads to the dissociation of casein micelles into smaller clusters. Compared with DSHP, TSC is known to have a higher affinity towards calcium (de Kort, 2012). Calcium sequestration exhibits greater affinity with citrate in comparison to orthophosphate, primarily due to the calcium's weaker association constant with HPO_4^{2-} (600 M^{-1}), as opposed to citrate's higher affinity (10^5 M^{-1}) for calcium (Kapoor, Metzger, Biswas & Muthukummarappan, 2007; Walstra & Jennes, 1984). Addition of TSC at 25 °C followed the same pattern as in DSHP, but with greater micellar dissociation and release of caseins at higher concentrations due to its higher affinity (Tables 3 and 4). This effect was further aggravated with an increase in temperature. Significant increase in particle size at 30 mM TSC and at 121 °C depicts further dissociation and significantly greater partial aggregation of likely released casein particles with intact casein micelles (Table 2).

The application of combined heat and shear resulted in further dissociation of casein micelles in DSHP-added dispersions. This dissociation may be explained by the interaction of CSS with structural elements in caseins, which is intensified by the applied heat and shear (Garcia et al., 2023). Applied heat and shear disrupt the mineral equilibrium between the serum and colloidal phase by causing changes in protein conformations, alterations to the micellar structure, and exposure of reactive sites inducing protein interactions (Table 2; Supplementary material Figs. S2A–C). On the other hand, TSC-added dispersions exhibited a gradual shear-dependant decline in both whey proteins and caseins indicating prominent shear-induced aggregation at both temperatures. TSC dissociates casein micelles by binding calcium from MCP to form non-sedimentable calcium citrate complexes. In addition, the increase in temperature may have resulted in the formation of κ-CN depleted porous micelles. Simultaneously, these micelles could have encountered shear-induced structural destabilisation, allowing for increased access to their interior and promoting aggregation. In addition to interacting with κ-CN, β-LG would also have been associated with α_{S2}-CN within the κ-CN depleted micelles through thiol-disulphide interchange forming complexes as evidenced by the particle size data which indicates partial aggregation at high shear rates. The shear-dependent substantial reduction of β-CN in the serum suggests potential self-association through hydrophobic linkages, likely attributable to the presence of distinct polar and hydrophobic domains, facilitated by the increased number of collisions (Walstra, 2001). The differences in casein solubilisation with applied shear were more prominent in MPC dispersions with added DSHP rather than TSC, as observed in SDS-PAGE. Generally, a marked increase in both β-CN and α_S-CN was observed at 20 mM, and further increase in concentration up to 30 mM led to a further surge in these caseins. MCP appears to be in the form of nanoclusters made up of small domains of calcium phosphate about 4 nm in diameter to which caseins are attached through their phosphoserine centres (De Kruif & Holt, 2003: Horne, 2006). Generally, caseins are differently phosphorylated with organic phosphate contents in the order of α_{S2}-CN > α_{S1}-CN > β-CN > κ-CN (Davies & Law, 1977; Swaisgood, 1992). The calcium-binding capacity of these proteins is mainly governed by their phosphorylation rate (Cross, Huq, Palamara, Perich & Reynolds, 2005). Such differences may account for the differential solubilisation of each casein. Even a low shear of 100 s^{-1} could aggravate a prominent solubilisation of caseins as access to MCP may be better due to shear-induced stretching of micelles.

Therefore, based on the current study, it appears that the effect of shear primarily depends on the calcium binding capacity of each CSS. As DSHP exhibits a lower affinity towards calcium, micelles are more susceptible to the impact of shear, especially at lower concentrations, as it has a lesser effect on micellar integrity. This observation is supported by the likely precipitation of calcium phosphate onto the micelles, which increases the surface area available for further interactions within the flow, thereby intensifying the shear impact. The deposition of calcium phosphate onto the micelle surface also shields the negative charge of the hairy layer of κ-CN, and this effect is amplified by the high temperature, leading to compromised heat stability. As a result, the reduction of electrostatic and steric repulsions causes the micelles to come closer together, resulting in higher particle collision rates under high shear conditions (Schokker & Dalgleish, 2000). Additionally, a higher concentration of phosphate can have a significant impact on the mineral equilibrium, affecting micellar integrity, and this effect is further exacerbated by shear. On the other hand, the relatively higher micellar dissociation caused by TSC resulted in the liberation of more caseins into the serum, making them more susceptible to the impact of shear.

5. Conclusion

The impact of shear appears predominantly influenced by the calcium binding capacity of the CSS. The substantial influence of shear on the casein micelles of dispersions containing DSHP suggests lower affinity towards calcium, which minimised micellar disruption, increased surface area for molecular collisions, and hydrodynamic fluid drag due to precipitation of calcium phosphate and subsequent lowering of electrostatic repulsions, resulting in bringing micelles closer and improving interactions. In contrast, higher affinity of TSC towards calcium disrupted micelles to a greater extent, liberating caseins and resulting shear induced aggregation of proteins.

The findings of this study may have substantial implications for the dairy and food processing industries. Understanding the role of CSS under controlled shear conditions offers the potential to enhance product quality, reduce the required amount of CSS for achieving desired outcomes, and optimise the physicochemical and functional properties of the final product. Therefore, shear, which is encountered frequently in dairy processing, should not be overlooked under given circumstances, as it has a direct impact on the properties of the final product, necessitating careful consideration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2023.105829.

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Supplementary material

Contents

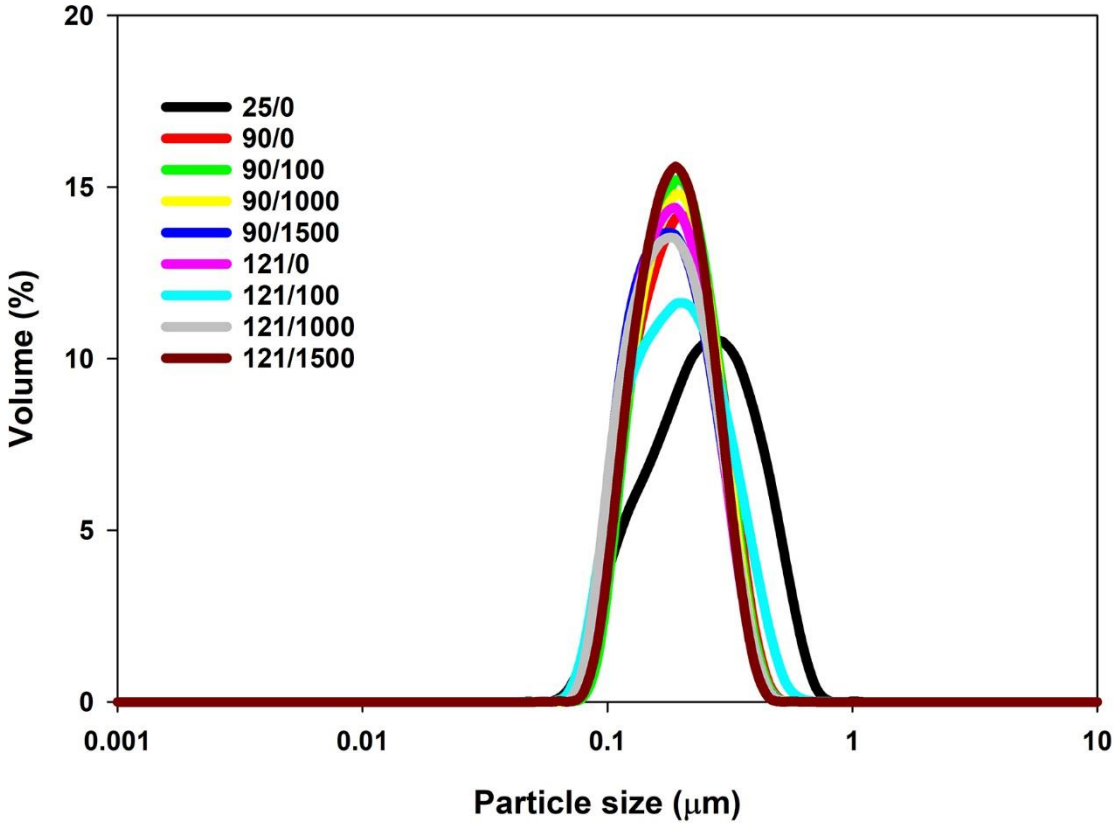

Fig. S1. Particle size distribution of 8% milk protein dispersion subjected to heating at 90 or 121 °C and shear rate of 100, 1000 or 1500 s^{-1}. The true controls were assessed prior to heating and during heating without shear (0 s^{-1}).

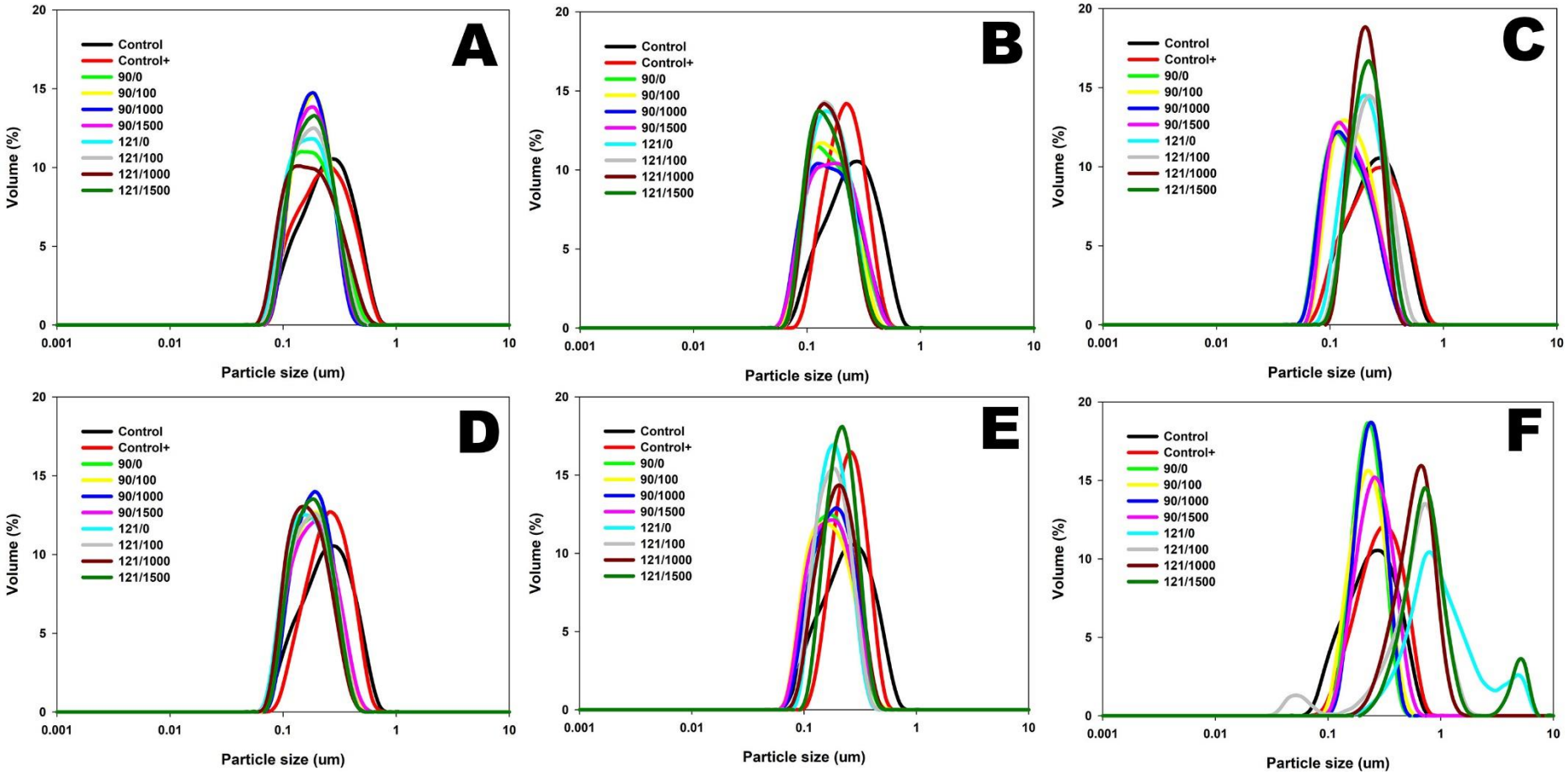

Fig. S2. Particle size distribution of 8% milk protein dispersions treated with 10 mM (A), 20 mM (B) and 30 mM (C) of disodium hydrogen phosphate or 10 mM (D), 20 mM (E) and 30 mM (F) of trisodium citrate under different temperatures (90 ℃ and 121 ℃) and shear rates (0 s^{-1}, 100 s^{-1}, 1000 s^{-1} and1500 s^{-1}).

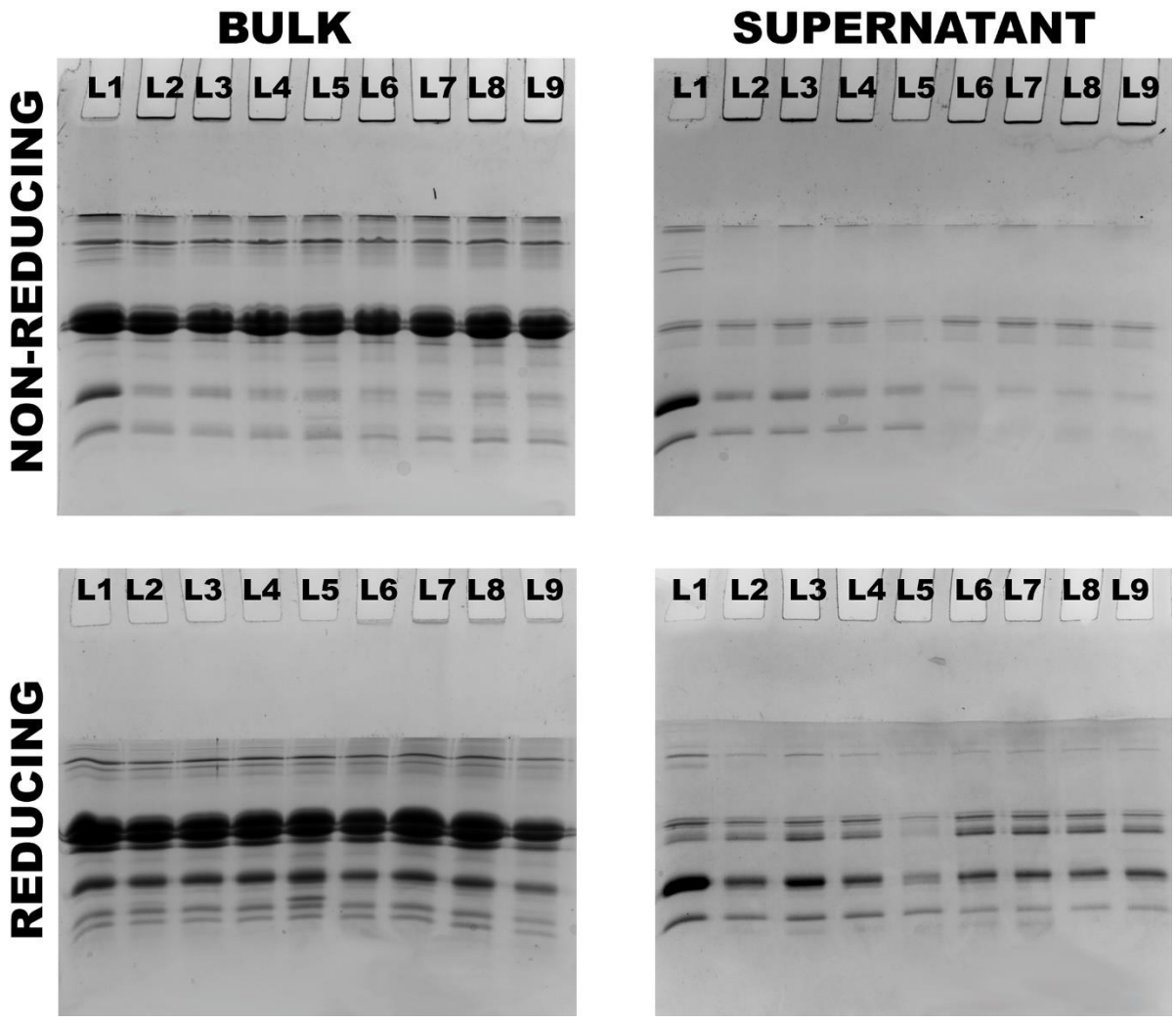

Fig. S3. Non-reducing and reducing SDS-PAGE analysis of bulk and supernatant MPC dispersions without the addition of calcium sequestering salts [lane sequence: (25-0)/90-0/90-100/90-1000/90-1500/121-0/-121-100/121-1000/121-1500 from left to right]

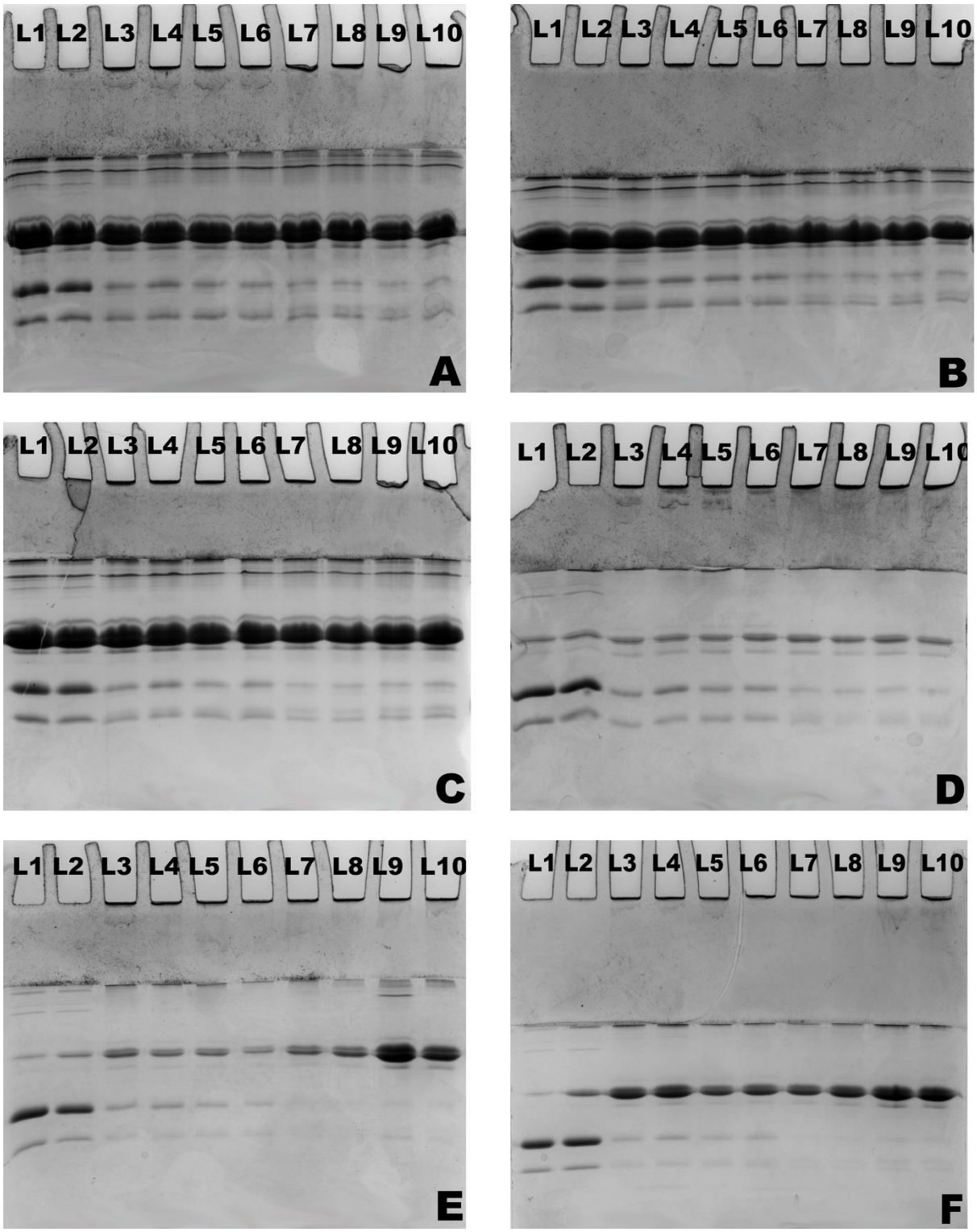

Fig. S4. Non-reducing SDS-PAGE analysis of bulk MPC dispersions (A–C) and corresponding supernatants (D–F) treated with disodium hydrogen phosphate at 10 mM (A,D), 20 mM (B,E) and 30 mM (C,F) [lane sequence: control without calcium sequestering salts (25-0)/25-0/90-0/90-100/90-1000/90-1500/121-0/-121-100/121-1000/121-1500 from left to right].

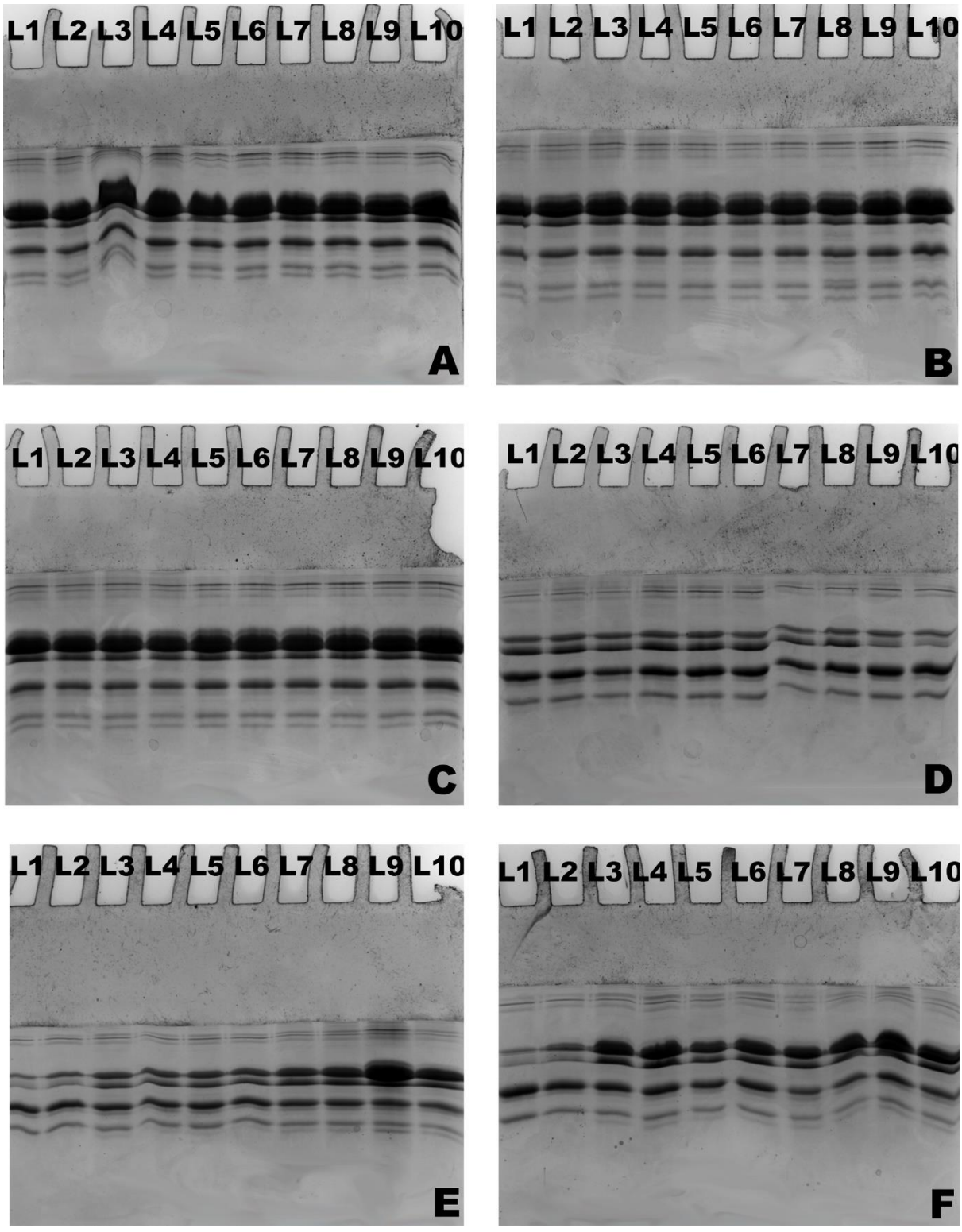

Fig. S5. Reducing SDS-PAGE analysis of bulk MPC dispersions (A–C) and corresponding supernatants (D–F) treated with disodium hydrogen phosphate at 10 mM (A,D), 20 mM (B,E) and 30 mM (C,F), 20 [lane sequence: control without calcium sequestering salts (25-0)/25-0/90-0/90-100/90-1000/90-1500/121-0/-121-100/121-1000/121-1500 from left to right].

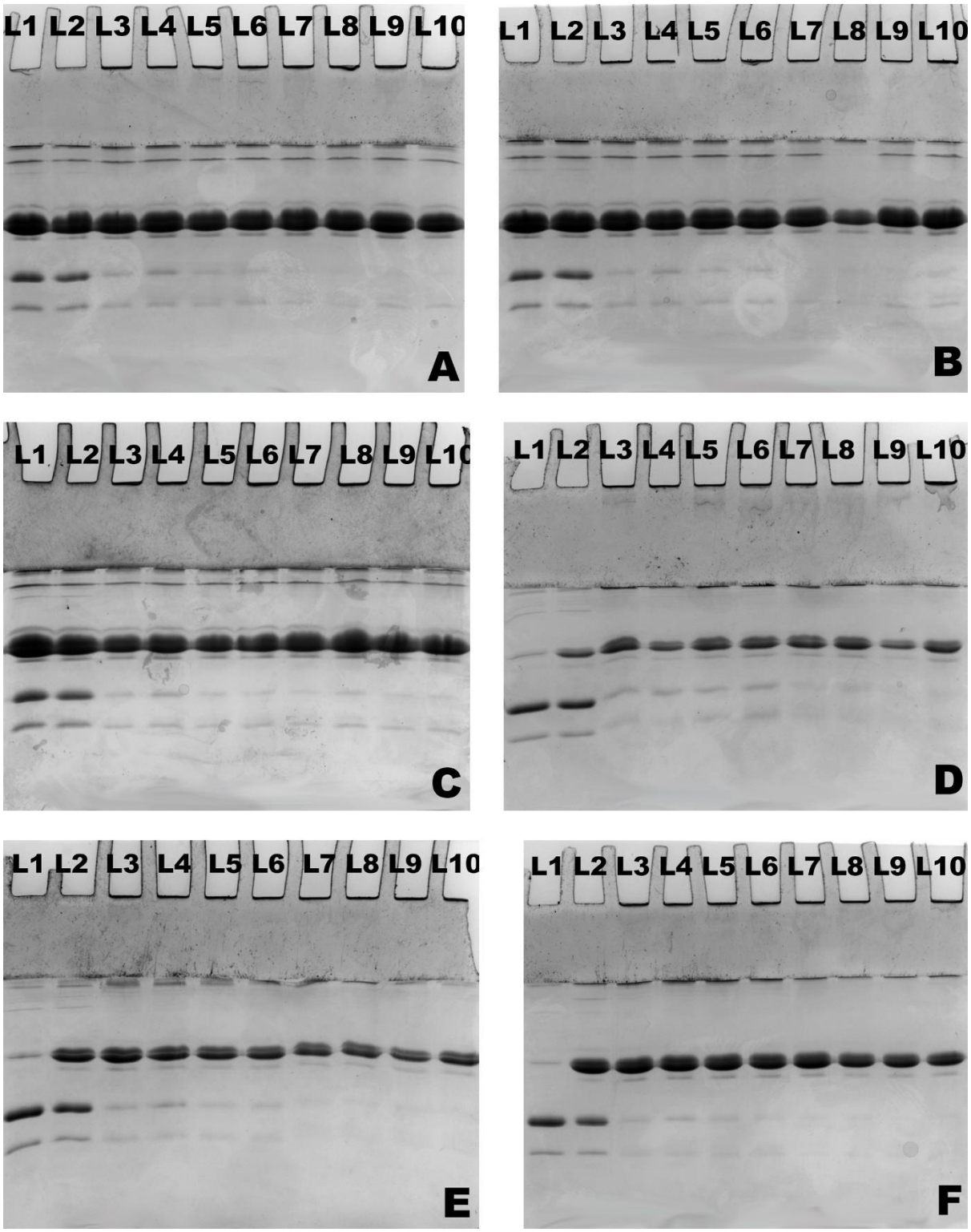

Fig. S6. Non-reducing SDS-PAGE analysis of bulk MPC dispersions (A–C) and the corresponding supernatants (D–F) treated with trisodium citrate at 10 mM (A,D), 20 mM (B,E) and 30 mM (C,F) [lane sequence: control without calcium sequestering salts (25-0)/25-0/90-0/90-100/90-1000/90-1500/121-0/-121-100/121-1000/121-1500 from left to right].

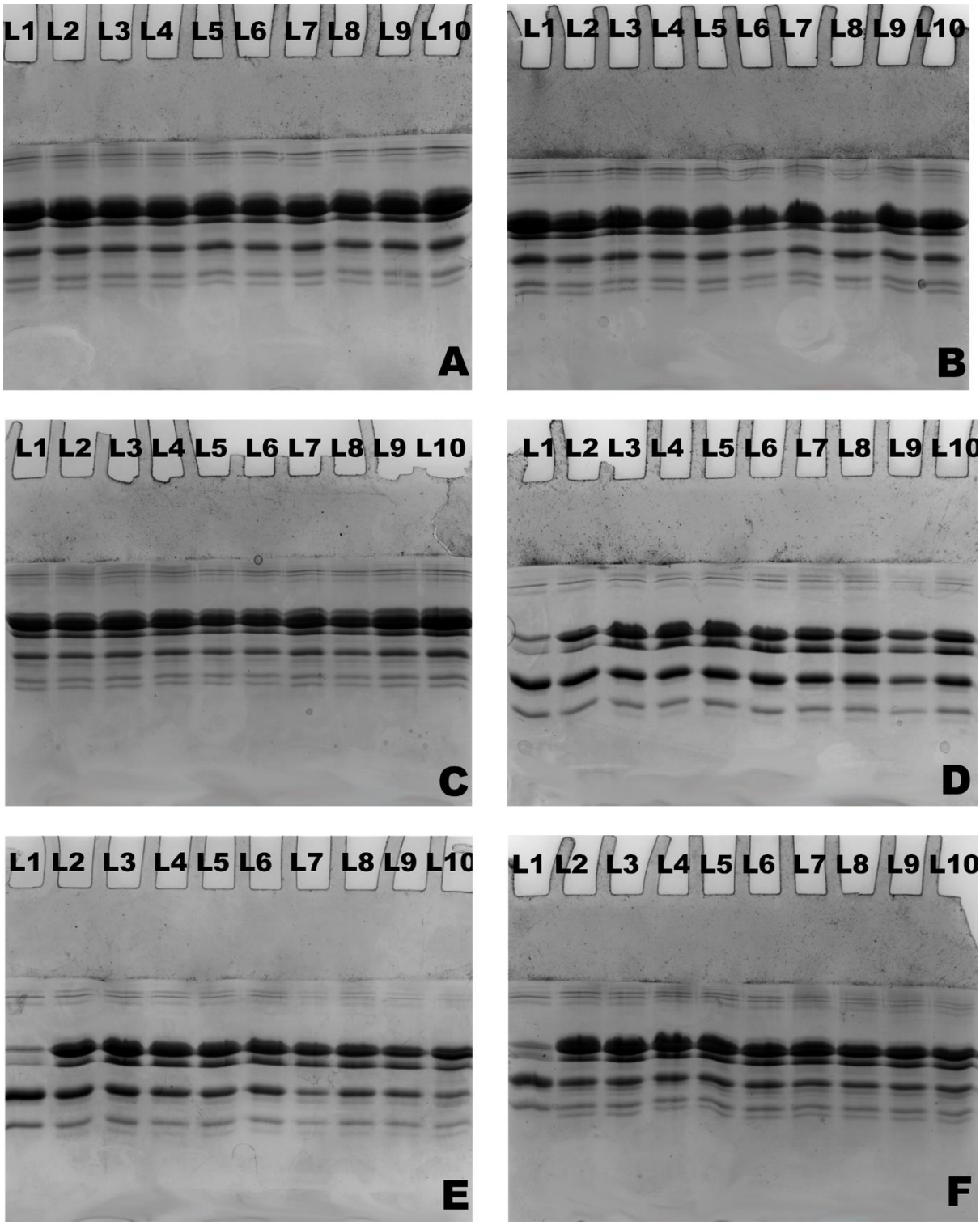

Fig. S7. Reducing SDS-PAGE analysis of bulk MPC dispersions (A–C) and the corresponding supernatants (D–F) treated with trisodium citrate at 10 mM (A,D), 20 mM (B,E) and 30 mM (C,F) [lane sequence: control without calcium sequestering salts (25-0)/25-0/90-0/90-100/90-1000/90-1500/121-0/-121-100/121-1000/121-1500 from left to right].

CHAPTER 6. Heat-Induced Changes in κ-Carrageenan-Containing Chocolate-Flavoured Milk Protein Concentrate Suspensions under Controlled Shearing

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Published in *Foods* 2023, 12, 4404

https://doi.org/10.3390/foods12244404

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Title of Paper/Journal/Book: Heat-Induced Changes in K-Carrageenan Containing Chocolate-Flavoured Milk Protein Concentrate Suspensions under Controlled Shearing

Surname: Mediwaththe | First name: Anushka

Institute: Institute for Sustainable Industries and Liveab | Candidate's Contribution (%): 80

Status:

Accepted and in press: ☐ Date:

Published: ☑ Date: 07/12

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Thom Huppertz	5	Designing experiments, manuscript editing and revision	[redacted]	14/02/2024
Jayani Chandrapala	5	Designing experiments, manuscript editing and revision	[redacted]	14/02/24
Todor Vasiljevic	10	Designing experiments, manuscript editing, revision and journal submission	[redacted]	14/02/24

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Heat-Induced Changes in κ-Carrageenan-Containing Chocolate-Flavoured Milk Protein Concentrate Suspensions under Controlled Shearing

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Abstract: Milk protein dispersions containing added cocoa powder (1.5% (*w*/*w*)) and sucrose (7% (*w*/*w*)) and varying levels of κ-carrageenan (0.01, 0.03, or 0.05% *w*/*w*) were subjected to combined heat treatment (90 °C/5 min or 121 °C/2.6 min) and shear (100 or 1000 s^{-1}) to investigate the heat stability of milk proteins. The application of shear led to a notable reduction in non-sedimentable proteins, resulting in an increase in the average particle size and apparent viscosity of the dispersions, particularly at high concentrations of k-carrageenan and elevated temperatures. This indicates that shear forces induced prominent protein aggregation, especially at higher κ-carrageenan concentrations. This aggregation was primarily attributed to the destabilisation of micelles and presence of loosely bound caseins within the κ-carrageenan network, which exhibited increased susceptibility to aggregation as collision frequencies increased due to shear.

Keywords: milk protein concentrate; heat stability; shear; κ-carrageenan; chocolate flavour

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Citation: Mediwaththe, A.; Huppertz, T.; Chandrapala, J.; Vasiljevic, T. Heat-Induced Changes in κ-Carrageenan-Containing Chocolate-Flavoured Milk Protein Concentrate Suspensions under Controlled Shearing. *Foods* **2023**, *12*, 4404. https://doi.org/10.3390/foods12244404

Academic Editor: Golfo Moatsou

Received: 14 November 2023
Revised: 1 December 2023
Accepted: 6 December 2023
Published: 7 December 2023

1. Introduction

In the marketplace, dairy beverages are available in diverse flavours, including chocolate, coffee, strawberry, and vanilla, as well as options with reduced or no fat. Among flavoured milk products, high-protein ready-to-drink (RTD) dairy beverages have gained popularity, designed for meal replacements and sports enthusiasts [1]. To achieve high protein content, milk protein concentrate (MPC) is commonly used, known for its substantial protein content ranging from 50% to 85% per dry solid content [2].

In chocolate-flavoured milk beverages, cocoa powder is often used as a flavouring agent. A significant proportion of cocoa powder particles remains insoluble when mixed with milk protein dispersions. As a result, during storage, some cocoa particles tend to settle at the bottom of the container due to the gravitational force [3]. This can be prevented by using hydrocolloid stabilizers such as κ-carrageenan. Such stabilizers create a gel network with milk protein that traps cocoa particles, reducing sedimentation, fat separation, and protein gelation [4].

The most recognised theory on the mechanism of κ-carrageenan's action in milk, specifically its interaction with casein micelles, involves the κ-carrageenan creating a weak gel, which holds the casein micelles suspended, preventing phase separation and increasing milk viscosity, aiding micellar stability, even below the critical gel concentration [5]. The carrageenan–milk protein interactions are influenced by the interactions among milk proteins themselves [6]. Heat treatment strengthens carrageenan gels in milk by displacing carrageenan complexed with κ-casein (CN) due to denatured β-lactoglobulin (LG). This

increases carrageenan availability for carrageenan–carrageenan interactions, enhancing long-term product stability [7]. Nevertheless, during sterilization, challenges such as undesired flocculation, coagulation, and sediment formation may arise due to increased viscosity and flavour–milk interactions [8]. The inappropriate use of stabilizers can lead to the manifestation of these undesirable characteristics in the final product.

Next to heat treatment, flavoured milk beverages with added κ-carrageenan undergo a range of processing stages, including pumping, blending, agitating, homogenizing, and passing through heat exchangers. These processes expose the beverages to hydrodynamic shear forces. These shear forces can induce structural changes of varying degrees to milk proteins [9]. While the combined effects of heating and shearing are commonly encountered in thermal processing, there is limited research on how these combined effects influence structural changes and protein interactions in complex milk systems, particularly in relation to milk protein concentrates (MPCs) during the production of chocolate flavoured high protein dairy beverages with varying levels of κ-carrageenan. Therefore, the objective of this study was to investigate the heat stability of chocolate-flavoured 8% MPC dispersion under controlled shearing at different heating regimes and varying levels of added κ-carrageenan. The processing conditions employed in this study closely resembled those used in commercial settings, such as a temperature of 90 °C for 5 min and 121 °C for 2.6 min and shear rates of 100 and 1000 s^{-1}. However, it is worth noting that lower heating rates were employed due to equipment limitations compared to industrial conditions.

2. Materials and Methods

2.1. Materials

MPC powder was procured from Fonterra Co-operative (Palmerston North, New Zealand) and stored in airtight plastic containers at a temperature of −20 °C. The composition of the MPC powder was 81.0% (*w*/*w*) total proteins, 1.6% (*w*/*w*) fat, 5.5% (*w*/*w*) carbohydrates, and 7.2% (*w*/*w*) ash, according to the manufacturer's specifications. All the chemicals used for the analysis were obtained from Sigma-Aldrich Pty Ltd. (Castle Hill, NSW, Australia). Ultrapure water (Milli-Q water by Merck Millipore in Bayswater, Vic, Australia) was used throughout the experiments.

2.2. Sample Preparation and Treatment

The MPC powder was reconstituted in Milli-Q water to obtain dispersions containing ~8% protein (*w*/*w*). The solution was continuously stirred for 1 h at 50 °C for the complete solubilisation of the powder [10]. A total of 1.5% cocoa powder (*w*/*w*) and 7% (*w*/*w*) sucrose were added to milk protein dispersions according to the typical composition of chocolate flavoured milk [11]. The κ-carrageenan concentration, selected from the recommended range commonly used in chocolate milk formulations [12], was 0.01%, 0.03%, or 0.05% (*w*/*w*) and was achieved by adding appropriate amount to the MPC dispersions prepared as mentioned above. The pH of all samples was adjusted to 6.8 via the slow addition of disodium phosphate buffer [4,13]. Samples were equilibrated at 20 °C for 1 h before the start of experiments.

Prepared chocolate-flavoured MPCs were heated at 90 °C for 5 min or 121 °C for 2.6 min at a constant shear rate (100 or 1000 s^{-1}) in a pressure cell (CC25/PR-150) of a rheometer (MCR 302e, Anton Paar GmbH, Graz, Austria) with a constant pressure of 250 kPa following the method of [14]. The heating was accomplished at a rate of 5 °C min^{-1} to the required temperature, followed by the required holding time and cooled down at a rate of 5 °C min^{-1}. Shear stress and viscosity were assessed at each treatment condition and the Reynolds number (Re) was calculated as reported previously [15]. The flow was characterised as the laminar for all conditions applying these equations.

The pH of each treated sample was measured immediately after treatment using a pH meter (WTW Inolab pH 720, Weilheim, Germany) at 20 °C and the first stable endpoint was recorded. A portion of the treated sample was centrifuged (Beckman Optima L-70 Ultracentrifuge, Indianapolis, IN, USA) at 100,000× *g* for 1 h at 20 °C. The super-

natant was carefully removed and used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.3. Rheological Measurements

The rheological data of untreated and rheometer-processed protein dispersions were collected using the above-used MCR 302e Rheometer (Anton Paar GmbH, Graz, Austria) and double-gap-cylinder measuring system (DG26.7-SN7721, Anton Paar, Graz, Austria). The viscosity measurements of the dispersions were collected via a programmed logarithmic shear rate ramp, increasing from 0.1 to 1000 s^{-1} for 50 points at 0.5 min intervals at 20 °C. The flow behaviour was described by the Ostwald de Waele model ($\tau = K\gamma^n$), where τ presents shear stress (Pa) and γ is shear rate (s^{-1}), while K and n are a consistency factor ($Pa{\cdot}s^n$) and the flow behaviour index, respectively.

2.4. Particle Size Measurements

Immediately after the treatment, particle size measurements were conducted using a Zetasizer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK), following the methodology outlined by [14]. Prior to the measurements, the treated samples were diluted 1000 times using skim milk ultra-filtrate prepared via the ultrafiltration of milk at 15 °C. This process employed a SEPA CF membrane module (Sterlitech Corporation, Kent, WA, USA) with a polyethersulfone (PES) membrane (190 × 140 mm) and a molecular cut-off of 10 kDa. The refractive indexes used in the calculations were 1.57 for MPC and 1.34 for SMUF, as previously reported [16].

2.5. Fourier Transform Infrared (FTIR) Analysis

The changes in the secondary structure of proteins were evaluated using an FTIR spectrometer (PerkinElmer Frontier FTIR Spectrometer, Waltham, MA, USA). FTIR spectra were acquired at room temperature (~20 °C) within 10 min after each treatment. Each spectrum was obtained by averaging 16 scans with a resolution of 4 cm^{-1} after subtracting the background [17]. To enhance resolution for qualitative analysis, the second derivative of all FTIR spectra within the broad Amide I region of 1700–1600 cm^{-1} was calculated using a PerkinElmer software (Spectrum 10 STD). Fourier self-deconvolution (FSD) and baseline correction were performed using an Origin Pro 2018 software (Origin Lab Corporation, Northampton, MA, USA) to identify prominent peaks corresponding to protein secondary structure within Amide I region. Peak fitting was carried out using the Gaussian function and peak fitting method, optimizing the fitting through iterative processes. The areas of the identified prominent peaks associated with specific secondary structures were summed and divided by the total area. This allowed for the determination of peak areas corresponding to five major protein secondary structures: α-helices (1660–1650 cm^{-1}), β-sheets (1637–1610 cm^{-1} and 1696–1680 cm^{-1}), random coils (1648–1638 cm^{-1}), and β-turns (1679–1667 cm^{-1}) [18,19]. The obtained results were then subjected to statistical analysis following the guidelines outlined in Section 2.7.

2.6. Sodium Dodecyl Sulphide Polyacrylamide Gel Electrophoresis (SDS PAGE)

After the treatments, all the treated samples, control samples, and the supernatants obtained from centrifuged samples (as mentioned earlier) were mixed with sodium dodecyl sulphate (SDS) sample buffer at a ratio of 1:25 (v/v). These mixtures were then stored at −20 °C until the time of electrophoresis. Both non-reducing and reducing SDS-PAGE (with β-mercaptoethanol as the reducing agent) were conducted following previously described methods [20]. Gel images were captured using Image Lab 5.1 software (Bio-Rad Laboratories, Galesville, NSW, Australia). The intensity of proteins in the treated samples, as observed in the supernatants through reducing gel electrophoresis, was expressed as a percentage of their corresponding proteins present in the control bulk dispersions.

2.7. Inductively Coupled Plasma Emission Spectrometric (ICP-OES) Analysis

For the determination of non-sedimentable minerals, samples were prepared by dissolving the ash obtained after combustion in a muffle furnace at 550 °C. This ash was dissolved in a mixture of 10 mL of 1 M HNO_3 acid and water to achieve a total solid (TS) content of 0.1%. Additionally, five standard solutions with varying concentrations of Ca, Mg, K, and P, ranging from 0.02% to 1% (*w*/*w*), were prepared following the method outlined in [21]. The non-sedimentable mineral content of both these standards and the supernatants after each treatment was then analysed using an inductively coupled plasma (ICP) atomic emission spectrometer (ICP E Multitype, Shimadzu Corporation, Kyoto, Japan), according to the procedure described by [22].

2.8. Statistical Analysis

Each experiment was performed in triplicate for both the control and treated chocolate-flavoured MPC dispersions, and statistical analyses were conducted using IBM SPSS Statistics software (version 28.0.1.0, IBM Corp., Armonk, NY, USA), employing a multivariate general linear model (GLM) protocol. The concentration of κ-carrageenan, temperature/time combinations, and shearing were considered the main factors in the analysis. The significance level was set at $p \leq 0.05$. Tukey's studentized range (HSD) test was used post hoc for multi-comparison of the means.

3. Results

3.1. Particle Size Distribution and Zeta Potential of MPC Suspensions upon Addition of κ-Carrageenan at Different Concentrations

The control dispersion had an average particle size of ~227 nm at 20 °C and decreased upon heating up to ~166 nm at 121 °C (Table 1). However, no significant change in zeta potential was observed (Table 1).

Table 1. Average particle size and zeta-potential of MPC suspensions with adjusted κ-carrageenan (κ-CG) concentrations subjected to different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

κ-CG (%)	Temp. (°C)	Average Particle Size (nm)			Zeta Potential (mV)		
		0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}
0	20	227 ^{C}			−20.8 ^{AB}		
	90	159 ^{Gb}	154 ^{Eb}	163 ^{Ea}	−24.4 ^{BCDa}	−22.8 ^{Ca}	−22.5 ^{Aa}
	121	166 ^{FGb}	171 ^{Da}	171 ^{Da}	−22.5 ^{ABCa}	−23.9 ^{Ca}	−22.0 ^{Aa}
0.01	20	211 ^{D}			−27.9 ^{D}		
	90	177 ^{EFa}	179 ^{Da}	185 ^{Ca}	−21.5 ^{ABCa}	−21.6 ^{BCa}	−20.9 ^{Aa}
	121	172 ^{EFGb}	199 ^{Ca}	173 ^{Db}	−20.5 ^{Aa}	−17.5 ^{Aa}	−20.0 ^{Aa}
0.03	20	269 ^{B}			−24.6 ^{CD}		
	90	179 ^{EFb}	197 ^{Ca}	167 ^{Dc}	−20.4 ^{Aa}	−21.6 ^{BCa}	−23.3 ^{Aa}
	121	177 ^{EFc}	226 ^{Aa}	209 ^{Ab}	−23.1 ^{ABCa}	−18.5 ^{ABa}	−21.4 ^{Aa}
0.05	20	297 ^{A}			−23.5 ^{ABC}		
	90	185 ^{Ec}	205 ^{Ba}	193 ^{Bb}	−22.6 ^{ABCb}	−21.7 ^{BCa}	−21.0 ^{Aa}
	121	182 ^{EFc}	203 ^{Ba}	198 ^{ABa}	−24.8 ^{CDb}	−22.9 ^{Cab}	−21.9 ^{Aa}
SEM *			2.57			−0.70	

The means in a column with different superscript upper-case letters and in a row with different superscript lower-case letters differ significantly ($p < 0.05$). * Standard error of mean.

The addition of κ-carrageenan to the MPCs at 20 °C resulted in an increase in average particle size from ~227 nm at the initial to ~297 nm at 0.05% κ-carrageenan concentration (Table 1). This size change can be attributed to the adsorption of κ-carrageenan onto the surface of casein micelles during the initial preparation of protein–polysaccharide

mixtures at an elevated temperature [23]. Furthermore, the addition of κ-carrageenan also induced a transition in the particle size distribution from monomodal to bimodal (Figure 1). This shift was characterized by a particle population that is relatively larger compared to dispersions without κ-carrageenan and appeared only at low and intermediate κ-carrageenan concentrations. The resultant particles were observed within the size range of both 0.1–1 μm and >1 μm. This could be indicative of a creation of a localised κ-carrageenan network that would entrap casein micelles [24], thus giving the appearance of larger particles. The zeta potential became more negative, from ~−20.8 mV in the absence of κ-carrageenan to ~−28 mV at 0.01% κ-carrageenan. When the concentration of κ-carrageenan was increased, zeta potential declined (e.g., ~−23.5 mV at 0.05% κ-carrageenan concentration) (Table 1). At low concentrations, κ-carrageenan may adsorb onto the surface of casein micelles, leading to an increase in the negative charge [23,24]. As κ-carrageenan concentrations increase, a network of κ-carrageenan molecules may form, trapping casein micelles within the network and resulting in a decreased negative charge and zeta potential [23].

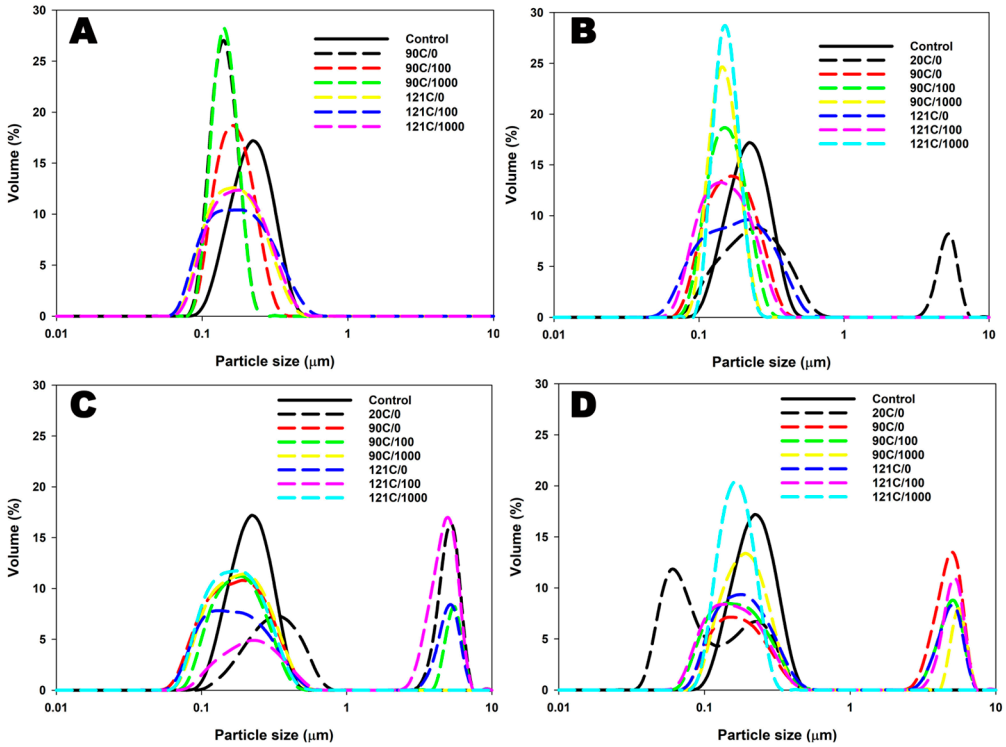

Figure 1. Particle size distribution of chocolate flavoured MPC suspensions without κ-carrageenan (**A**) and with κ-carrageenan concentrations of 0.01% (**B**), 0.03% (**C**), and 0.05% (**D**) processed under different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

Particle sizes in heated dispersions with added κ-carrageenan decreased across all concentrations (e.g., from ~297 nm at 20 °C to ~182 nm at 121 °C at 0.05%). However, the resulting particles were comparatively larger than particles without the stabilizer (Table 1). This change occurred in a concentration-dependent manner, with higher concentrations leading to larger particles (Figure 1 and Table 1). Consequently, zeta potential values also became less negative in all heated dispersions.

When subjected to combined heating and shearing, no significant changes in particle size or zeta potential were observed in control dispersions without κ-carrageenan (Figure 1A and Table 1). However, the addition of κ-carrageenan to the dispersions and subsequent exposure to heat and shear resulted in a more uniform size distribution of particles, with an increase in the average particle size as the shear rate increased (e.g., ~182 nm at 0 s^{-1} to ~198 nm at 1000 s^{-1} at 121 °C and at 0.05%) (Figure 1 and Table 1). Particles ranging

between 0.1 and 1 μm were observed in MPCs without κ-carrageenan. When the shear rate was increased up to 1000 s^{-1} in the dispersions with added κ-carrageenan, the proportion of particles within the size range of 0.1–1 μm was increased compared to 100 s^{-1} (Figure 1). In contrast, at 100 s^{-1}, there was a relatively larger proportion of particles observed >1 μm. This suggests that at 1000 s^{-1}, a higher number of casein particles did not interact with κ-carrageenan or aggregates formed were fragmented due to shear, while at 100 s^{-1}, more interactions occurred between caseins and κ-carrageenan. This implies that the interaction between casein micelles and κ-carrageenan may be influenced by the applied shear rate (Figure 1 and Table 1).

Specifically, at a shear rate of 1000 s^{-1}, aggregates within the larger particle size range were considerably disrupted, leading to the generation of particles with more uniform sizes. This effect was particularly pronounced at lower concentrations of κ-carrageenan (0.01% and 0.03%) and at a temperature of 121 °C (Figure 1 and Table 1). At elevated concentrations, κ-carrageenan has the ability to create a robust polysaccharide network in which casein micelles become physically entrapped [24,25]. However, the stability of this κ-carrageenan network is influenced by the amount of κ-carrageenan present. When subjected to cooling, the shear forces applied can disrupt the formation of the κ-carrageenan network, leading to its destabilization and potential breakdown.

3.2. Mineral Distribution of MPCs upon Addition of κ-Carrageenan at Different Concentrations

Ca, Mg, K, and P collectively constitute a significant fraction of mineral constituents in milk. These ions exhibit diverse associations among themselves and with milk proteins. Notably, K demonstrates full solubility, whereas Ca, Mg, and P exhibit partial association with casein micelles [26]. The partitioning of these elements between non-sedimentable and micellar phases markedly influences the stability of the colloidal dispersion in milk [27].

The control dispersion at 20 °C contained ~5.84 mM of non-sedimentable calcium. Heating control dispersions primarily resulted in a decrease in the levels of non-sedimentable calcium up to ~4.96 mM at 121 °C, as it precipitated as calcium phosphate due to the applied heat (Table 2). However, this reduction was not as prominent as in κ-carrageenan-added dispersions.

As depicted in the table, Mg content within the MPC suspensions exhibited notable elevation, likely attributable to the inclusion of cocoa [28]. Upon the addition of κ-carrageenan at 20 °C, there was a significant reduction in the levels of non-sedimentable minerals, including Ca, Mg, and P (Table 2). Furthermore, as the concentration of κ-carrageenan increased, these mineral levels within the supernatant decreased even further (e.g., Ca content decreased from ~5.84 mM at 20 °C in the absence of κ-carrageenan to ~2.37 mM at 0.05% concentration of κ-carrageenan) (Table 2). This reduction in non-sedimentable mineral content can be attributed to the interaction between the κ-carrageenan and minerals in milk and interactions with proteins, which can impact the distribution and availability of minerals within the dispersions. This effect appeared to be augmented with the application of heat, as it further reduced Ca, K, and P from the soluble phase, likely due to heat-induced precipitation or involvement in the formation of a CN/κ-carrageenan network (e.g., Ca content was further decreased from ~2.37 nm at 20 °C to ~1.97 nm at 121 °C and at 0.05%) [29].

The application of combined heat and shear to the dispersions without κ-carrageenan resulted in an increase in all the investigated minerals in the soluble phase, including Ca, K, Mg, and P with a comparatively greater incline in both Ca and K (Table 2). In the dispersions containing κ-carrageenan, the impact of shear on minerals appears to be dependent on the concentration of κ-carrageenan at both temperatures. At a low concentration of κ-carrageenan (0.01%), the combined application of heat and shear resulted in an increase in all non-sedimentable minerals with a >50% increase in Ca, K, and P levels at 1000 s^{-1} and at 121 °C (Table 2). However, as the concentration of κ-carrageenan increased, there was either a slight decline or no substantial change in the levels of non-sedimentable minerals (Table 2). The decline in minerals within the soluble phase may be attributed to

the enhanced stability of the CN-κ-carrageenan network formed with the involvement of minerals in the presence of higher κ-carrageenan concentrations, which is less susceptible to disruption by shear forces.

Table 2. Mineral concentration in supernatants of MPC suspensions with adjusted κ-carrageenan concentrations subjected to different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

κ-CG (%)	Temp (°C)	Mineral Concentration (mM)											
		Ca			K			Mg			P		
		0 s^{-1}	**100 s^{-1}**	**1000 s^{-1}**	**0 s^{-1}**	**100 s^{-1}**	**1000 s^{-1}**	**0 s^{-1}**	**100 s^{-1}**	**1000 s^{-1}**	**0 s^{-1}**	**100 s^{-1}**	**1000 s^{-1}**
0	20	5.84 ^{A}			10.19 ^{D}			17.05 ^{A}			7.47 ^{A}		
	90	5.76 ^{Ac}	7.45 ^{Ab}	7.85 ^{Aa}	9.27 ^{Eb}	9.61 ^{Db}	10.58 Ea	16.54 Ab	18.10 Aa	18.33 Aa	6.84 ABb	9.06 ^{Aa}	9.23 ^{Aa}
	121	4.96 ^{Bc}	6.68 ^{Bb}	7.29 ^{Ba}	8.97 ^{Fb}	7.79 F^{c}	14.73 Aa	16.00 Bc	17.21 Bb	18.57 Aa	6.64 ABb	8.67 ^{Aa}	8.30 ^{Ba}
0.01	20	4.08 ^{C}			12.87 ^{A}			14.56 ^{C}			6.26 ABC		
	90	3.70 ^{Cb}	3.75 ^{Db}	3.83 ^{Db}	10.62 Cc	11.10 Bb	12.40 Ba	14.93 Cb	15.15 Dab	15.60 Ca	4.87 DEb	5.59 ^{Ca}	6.10 ^{Ca}
	121	3.11 ^{Db}	4.82 ^{Ca}	4.81 ^{Ca}	6.31 ^{Gc}	9.40 ^{Db}	10.19 Fa	16.58 b	16.58 Cb	17.22 Ba	4.57 DEc	6.38 ^{Bb}	8.53 ABa
0.03	20	3.70 ^{C}			11.30 ^{B}			13.79 ^{D}			6.06 BCD		
	90	3.60 ^{Ca}	3.42 Dab	2.92 ^{Eb}	12.84 Aa	10.21 Cc	11.18 Cb	16.93 Aa	16.54 Cab	10.21 Cb	5.80 CDa	5.02 ^{Db}	5.35 ^{Cb}
	121	2.63 ^{Eb}	3.01 ^{Da}	2.80 Eab	12.58 Aa	12.02 Ab	10.79 Dc	14.54 Cb	15.07 Da	14.44 Db	5.92 BCDa	5.89 ^{Ca}	5.37 ^{Cb}
0.05	20	2.37 ^{EF}			12.23 ^{A}			13.67 ^{D}			5.01 CDE		
	90	2.34 EFa	2.30 ^{Ea}	2.10 ^{Fa}	11.58 Ba	11.19 Bab	10.87 Db	14.65 Cb	15.38 Da	14.60 Db	5.06 CDEa	4.87 ^{Ea}	4.21 ^{Db}
	121	1.97 ^{Fa}	1.77 ^{Fa}	1.56 ^{Ga}	9.52 ^{Ea}	8.70 ^{Eb}	8.23 ^{Gc}	13.93 Da	12.85 Eb	12.49 Eb	3.84 ^{Ea}	3.14 ^{Fb}	2.22 ^{Ec}
SEM *		0.05			0.08			0.12			0.07		

The means in a column with different superscript upper-case letters and in a row with different superscript lower-case letters differ significantly ($p < 0.05$). * Standard error of mean.

3.3. Rheological Properties of MPCs upon Addition of κ-Carrageenan at Different Concentrations

Both non-heated and heated control dispersions display shear-thinning behaviour (characterised by $n < 1$), signifying that apparent viscosity decreases as shear rate increases. With heating, the consistency factor decreased, leading to lower apparent viscosity (Table 3).

In the presence of κ-carrageenan, all dispersions exhibited shear-thinning behaviour, regardless of the treatment they underwent, as indicated by $n < 1$ in all κ-carrageenan-added dispersions. The addition of κ-carrageenan resulted in an increase in apparent viscosity, as evidenced by an elevated consistency factor (Figure 2 and Table 3). This increase in viscosity can be attributed to the linear macromolecular structure and polyelectrolytic properties of carrageenan, which promotes interactions with proteins and minerals. Consequently, apparent viscosities were observed to increase with higher κ-carrageenan concentrations. The highest apparent viscosity was observed at a κ-carrageenan concentration of 0.05% (Table 3). When dispersions containing added κ-carrageenan were heated, a decrease in the consistency factor was observed across all three concentrations (Table 3). This decrease was notably more pronounced after treatment at 90 °C compared to 121 °C.

Table 3. Consistency factor (K) and flow behaviour index (n) of MPC suspensions with adjusted κ-carrageenan concentrations subjected to different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

κ-CG (%)	Temp. (°C)	Consistency Factor ($Pa.s^n$)			Flow Behaviour Index (-)		
		0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}
0	20	0.011 ^{HI}			0.91 ^{B}		
	90	0.006 ^{IJb}	0.007 ^{Gab}	0.008 ^{Fa}	0.98 ^{Aa}	0.98 ^{Aa}	0.97 ^{Aa}
	121	0.005 ^{Jb}	0.006 ^{Ga}	0.007 ^{Fa}	0.96 ^{Aa}	0.96 ^{Aa}	0.95 ^{Ba}
0.01	20	0.028 ^{G}			0.82 ^{D}		
	90	0.014 ^{Ha}	0.014 ^{Fa}	0.015 ^{Ea}	0.87 ^{Ca}	0.87 ^{Ba}	0.86 ^{Ca}
	121	0.021 ^{Gb}	0.022 ^{Eb}	0.028 ^{Da}	0.84 ^{Da}	0.84 ^{Ca}	0.81 ^{Db}
0.03	20	0.170 ^{E}			0.65 ^{F}		
	90	0.089 ^{Fc}	0.103 ^{Db}	0.119 ^{Ca}	0.67 ^{Ea}	0.67 ^{Da}	0.67 ^{Ea}
	121	0.236 ^{Dc}	0.370 ^{Bb}	0.641 ^{Ba}	0.60 ^{Ga}	0.61 ^{Ga}	0.50 ^{Fb}
0.05	20	0.763 ^{A}			0.49 ^{I}		
	90	0.243 ^{Cc}	0.367 ^{Cb}	0.709 ^{Aa}	0.58 ^{Ga}	0.57 ^{Fa}	0.47 ^{Gb}
	121	0.622 ^{Bc}	0.634 ^{Ab}	0.641 ^{Ba}	0.51 ^{Ha}	0.51 ^{Ga}	0.46 ^{Fb}
SEM *			0.0017			0.0061	

The means in a column with different superscript upper-case letters and in a row with different superscript lower-case letters differ significantly ($p < 0.05$). * Standard error of mean.

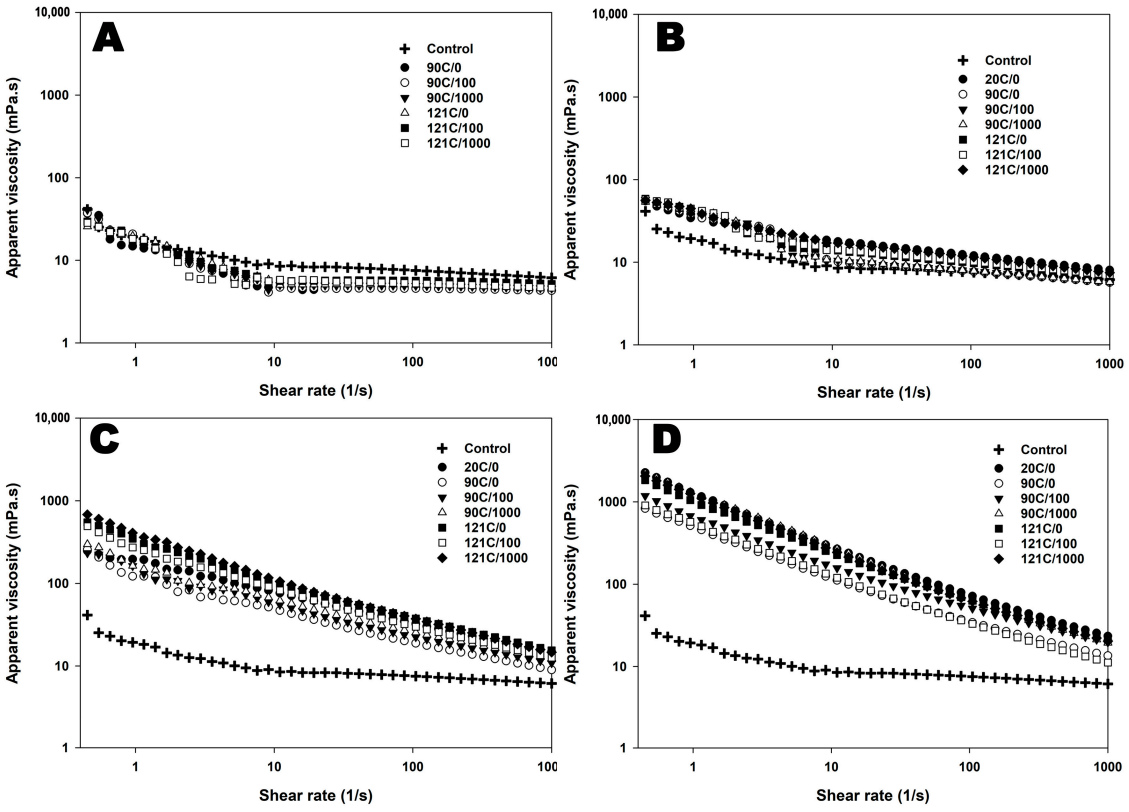

Figure 2. Apparent viscosity of chocolate flavoured MPC suspensions as a function of shear rate (0, 100, or 1000 s^{-1}) applied during heating at 90 °C for 5 min or 121 °C for 2.6 min at 0% κ-carrageenan (**A**), 0.01% κ-carrageenan (**B**), 0.03% κ-carrageenan (**C**), or 0.05% κ-carrageenan (**D**). The viscosity measurements were completed after the treatments at 20 °C.

As observed with non-heated and heated controls, the combined application of heat and shear to controls resulted in the system that exhibited shear-thinning behaviour (indicated by $n < 1$) with an increase in the consistency factor as shear rate increased, signifying an increase in viscosity (Figure 2 and Table 3). Similarly, all κ-carrageenan-added dispersions exhibited shear-thinning behaviour ($n < 1$) under the influence of combined heat and shear. The changes in the consistency factor were dependent on the concentration of κ-carrageenan. In a general trend, elevated temperatures and increased shear rates led to higher consistency factors, consequently elevating the apparent viscosity of all κ-carrageenan-added dispersions (Figure 2 and Table 3). At higher concentrations of κ-carrageenan, the formation of a more robust κ-carrageenan–casein network, resistant to shear forces, ultimately contributed to the increased apparent viscosity of these dispersions.

3.4. Interactions and Aggregation of Proteins as Observed by SDS-PAGE Analysis upon the Addition of κ-Carrageenan at Different Concentrations

In the control dispersions, an increase in temperature resulted in a decline of β-LG and α-LA levels in the soluble phase due to aggregation and precipitation. On the other hand, all α_s-, β-, and κ-CN levels increased in the soluble phase, depicting the dissociation of caseins with the increase in temperature (Supplementary Materials, Figure S1 and Table 4).

Table 4. Intensity of caseins and whey proteins in supernatants as a proportion (%) of their intensity in the control bulk suspensions subjected to different treatments resolved under reducing electrophoretic conditions and quantified using a ChemiDoc imager.

κ-CG (%)	Temp. (°C)	α_s-CN			β-CN			κ-CN			β-LG			α-LA		
		0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}
0	20	4.5 CD			8.9 CD			43.6 H			99.5 A			98.9 A		
	90	4.7 BCb	6.4 Aa	6.7 Aa	12.0 Bb	14.5 Ba	14.9 Ba	44.7 Gb	47.7 Ea	48.1 Da	75.3 Hb	80.4 Aa	80.9 Aa	85.3 Cc	88.5 Aa	87.1 Ab
	121	4.8 Bb	5.3 Bab	5.9 Aa	16.4 Ac	17.7 Ab	23.7 Aa	48.3 Ec	51.6 Db	53.8 Ba	68.1 Kc	75.2 Cb	77.2 Ca	63.1 Kc	75.9 Cb	76.8 Ba
0.01	20	4.0 E			8.4 D			32.6 I			97.4 B			94.4 B		
	90	4.7 BCa	4.2 Cbc	3.9 Cc	9.0 CDa	7.7 Db	5.7 Dc	59.6 Ba	57.1 Cb	49.6 Cc	82.6 Fa	80.7 Ab	79.9 Bc	80.1 Ea	80.3 Ba	75.4 Bb
	121	5.3 Ab	5.6 Ba	4.5 BCc	9.7 Ca	8.9 Cb	8.0 Cc	64.4 Aa	46.7 Fb	16.8 Hc	73.5 Ia	66.8 Eb	60.2 Gc	73.6 Ga	53.2 Gb	45.2 Gc
0.03	20	3.6 F			5.5 FG			16.6 J			95.9 C			82.7 D		
	90	4.3 Da	3.9 Cb	3.3 Cc	6.3 EFa	6.1 Eab	5.7 Db	64.4 Ab	66.8 Aa	60.7 Ac	87.3 Da	77.3 Bb	74.8 Dc	77.7 Fa	75.5 Cb	71.0 Cc
	121	5.4 Aa	5.6 Ba	4.9 Bb	7.2 Ea	6.7 Eb	5.9 Dc	53.8 Cb	60.6 Ba	36.6 Ec	75.7 Ha	74.8 Cb	69.5 Fc	66.7 Ib	68.8 Da	64.9 Dc
0.05	20	2.1 I			3.6 H			16.0 K			84.3 E			64.2 J		
	90	2.5 Ha	2.2 Eb	2.0 Eb	4.0 Ha	3.9 Ga	3.1 Fb	47.6 Fa	43.7 Gb	33.2 Fc	79.5 Ga	75.1 Cb	70.4 Ec	71.2 Ha	63.8 Eb	58.9 Ec
	121	3.3 Ga	3.1 Dab	2.7 Db	5.1 Ga	4.9 Fa	4.3 Eb	49.8 Da	42.4 Hb	22.5 Gc	72.1 Ja	67.9 Db	60.7 Gc	62.1 La	55.5 Fb	54.3 Fc
SEM *			0.081			0.278			0.133			0.158			0.163	

The means in a column with different superscript upper-case letters and in a row with different superscript lower-case letters differ significantly ($p < 0.05$). * Standard error of mean.

In the dispersions containing added κ-carrageenan, it was observed that the levels of both whey proteins and caseins in the serum phase decreased with increasing κ-carrageenan concentration at 20 °C, consistent with findings from previous studies (Supplementary Materials, Figure S1 and Table 4) [30]. This can be attributed to the formation of a protein-κ-carrageenan network, which sedimented upon ultracentrifugation.

Heating MPCs with added κ-carrageenan substantially increased the levels of non-sedimentable caseins (particularly κ-CN), probably due to detachment of κ-CN/κ- carrageenan complexes from casein micelles and the destabilisation of micelles. However, soluble whey proteins in the supernatant declined further, probably due to heat-induced denaturation and aggregation (Supplementary Materials, Figure S1 and Table 4).

In the dispersions without added κ-carrageenan, the combined application of heat and shear gradually increased the levels of all β-LG, α-LA, and κ-CN in the soluble phase compared to heated dispersions at both 90 and 121 °C. This increase indicates the shear-induced fragmentation of aggregates. Additionally, both α_s- and β-CN levels in the soluble phase also increased with a notably higher concentration of β-CN, reaching their highest levels at 1000 s^{-1} and 121 °C, likely due to the shear-induced structural destabilization of caseins (Supplementary Material Figure S1 and Table 4). On the other hand, in all κ-carrageenan-added dispersions, and the combined application of heat and shear decreased the levels of all β-LG, α-LA, and κ-CN in the soluble phase, indicating the precipitation of these proteins upon centrifugation. All these proteins appeared to decrease in a concentration-dependent manner, reaching their lowest levels at a κ-carrageenan concentration of 0.05% (Supplementary Materials, Figure S1 and Table 4).

3.5. Conformational Properties of MPC System with Added k-Carrageenan

In the control dispersions, heating resulted in a notable reduction in the peak intensity at ~1637 cm^{-1} (Figure 3) and a significant decrease in its content (Table 5), indicating a decline in intramolecular β-sheets. Furthermore, prominent peaks at ~1671 cm^{-1} and ~1674 cm^{-1} were observed (Figure 3) with a significant increase in their peak areas (Table 5), suggesting a concurrent increase in β-turns.

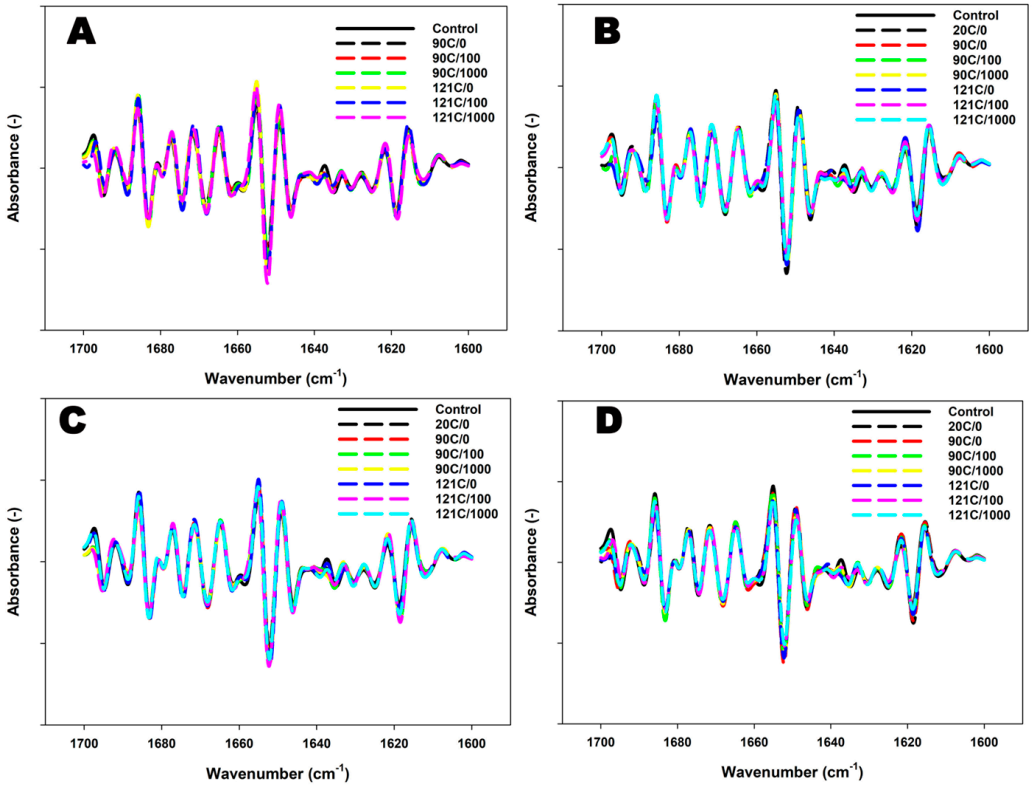

Figure 3. FTIR spectra (second derivative) for the Amide I region of chocolate-flavoured MPC suspensions without κ-carrageenan (**A**) and with κ-carrageenan concentrations of 0.01% (**B**), 0.03% (**C**), and 0.05% (**D**) processed under different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

The addition of κ-carrageenan at 20 °C resulted in reduced peak intensities, particularly at ~1655 cm^{-1} and ~1637 cm^{-1}, and reduced peak areas, indicating a decrease in α-helical structures and intramolecular β-sheets in a concentration-dependant manner in the MPC system, respectively (Figure 3, Table 5). Simultaneously, an increase in random coil structures was observed, represented by intense peaks at ~1642 cm^{-1} and its content (Figure 3, Table 5). Heating of κ-carrageenan-added dispersions led to a further reduction in peak intensity at both ~1655 cm^{-1} and ~1637 cm^{-1} (Figure 3), along with a decrease in their content (Table 5), indicating a decline in α-helical structures and intramolecular β-sheets, respectively. Simultaneously, an increase in peak intensities at ~1672 cm^{-1} was observed, suggesting an increase in β-turns. These changes appeared to be moderated with the further addition of κ-carrageenan (Figure 3 and Table 5).

The combination of heat and shear applied to MPCs without added κ-carrageenan further reduced intramolecular β-sheets while increasing β-turns, as indicated by the reduction in peak intensities at ~1637 cm^{-1}, ~1622 cm^{-1}, and their respective content, along with an increase in peak intensity at ~1672 cm^{-1} and its content (Figure 3 and Table 5). Additionally, a decline in α-helix content and a simultaneous increase in random coils were also observed (Table 5). Furthermore, the aggregated β-sheets were also decreased, as observed (Figure 3, Table 5). The combined application of heat and shear to κ-carrageenan-added dispersions resulted in a reduction in peak intensity at ~1637 cm^{-1} and its content, depicting a gradual decline in intramolecular β-sheets. There was an increase in peak intensity within the ~1682–1700 cm^{-1} region in all κ-carrageenan-added dispersions, along with its content, depicting an increase in aggregated β-sheets. The number of β-turns gradually declined as observed in the reduction in peak intensity at ~1672 cm^{-1} and its content (Figure 3 and Table 5). This decrease was most prominent at the highest concentration of κ-carrageenan. Additionally, there was a reduction in peak intensity at ~1655 cm^{-1}, along with its content, indicating a reduction in α-helical structures (Figure 3 and Table 5). Simultaneously, an increase in random structures was also observed along with the increased heat and shear (Table 5).

Table 5. Total percentage areas of different secondary structures in the Amide I region of protein milk dispersions with adjusted κ-carrageenan concentrations subjected to different temperatures (20, 90, or 121 °C) and shear rates (0, 100, or 1000 s^{-1}).

κ-Carrageenan Addition (%)	Temp. (°C)	Intramolecular β-Sheets (1615–1637)			Random Coils (1638–1645)			α-Helix (1646–1664)			β-Turns (1665–1681)			Aggregated β-Sheets (1682–1700)		
		0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}	0 s^{-1}	100 s^{-1}	1000 s^{-1}
0	20	50.41 ^{A}			6.41 ^{H}			31.01 ^{A}			8.15 ^{I}			3.51 ^{J}		
	90	38.89 ^{Da}	31.45 ^{Bb}	25.49 ^{Bc}	5.87 ^{Ic}	7.87 ^{Eb}	11.05 ^{Fa}	22.59 ^{Ha}	17.62 ^{Eb}	12.87 ^{Ec}	24.62 ^{Dc}	25.71 ^{Bb}	30.41 ^{Ba}	5.13 ^{Ia}	4.72 ^{Fb}	3.33 ^{Hc}
	121	33.93 ^{Ha}	31.94 ^{Bb}	25.14 ^{Bc}	8.06 ^{Fc}	9.17 ^{Db}	12.17 ^{Da}	13.02 ^{Ja}	12.95 ^{Ga}	11.70 ^{Fb}	31.39 ^{Ac}	33.09 ^{Ab}	37.43 ^{Aa}	6.82 ^{Fa}	5.54 ^{Eb}	4.14 ^{Gc}
0.01	20	43.88 ^{B}			10.47 ^{A}			29.20 ^{B}			16.45 ^{H}			5.84 ^{H}		
	90	30.51 ^{Ia}	28.53 ^{Eb}	23.85 ^{Dc}	8.00 ^{Gc}	9.73 ^{Bb}	11.35 ^{Ea}	20.07 ^{Ia}	16.25 ^{Fb}	10.03 ^{Gc}	18.35 ^{Ga}	17.49 ^{Fb}	16.12 ^{Ec}	6.18 ^{Gc}	6.74 ^{Db}	7.81 ^{Fa}
	121	23.74 ^{La}	23.73 ^{Fa}	19.86 ^{Fb}	3.60 ^{Jc}	6.19 ^{Fb}	11.12 ^{Fa}	10.27 ^{Ka}	9.27 ^{Hb}	7.78 ^{Hc}	19.39 ^{Fa}	18.91 ^{Eb}	17.23 ^{Dc}	7.82 ^{Cc}	8.18 ^{Cb}	9.32 ^{Ea}
0.03	20	39.01 ^{C}			10.14 ^{B}			28.16 ^{C}			19.61 ^{F}			6.73 ^{F}		
	90	35.03 ^{Ga}	33.46 ^{Ab}	24.08 ^{Cc}	8.14 ^{Ec}	9.54 ^{Cb}	12.29 ^{Da}	24.63 ^{Fa}	21.04 ^{Db}	19.63 ^{Dc}	24.63 ^{Da}	19.96 ^{Db}	17.51 ^{Dc}	7.92 ^{Cc}	8.92 ^{Bb}	10.01 ^{Ba}
	121	30.90 ^{Ia}	29.46 ^{Db}	23.06 ^{Ec}	3.49 ^{Kc}	9.59 ^{Cb}	12.97 ^{Ca}	25.08 ^{Ea}	24.79 ^{Aa}	22.28 ^{Ab}	26.67 ^{Ca}	24.16 ^{Cb}	19.67 ^{Cc}	8.17 ^{Bc}	8.99 ^{Bb}	9.52 ^{Da}
0.05	20	37.74 ^{E}			10.13 ^{B}			26.67 ^{D}			18.45 ^{G}			7.15 ^{E}		
	90	36.02 ^{Fa}	30.11 ^{Cb}	27.75 ^{Ac}	9.25 ^{Cc}	10.46 ^{Ab}	13.76 ^{Ba}	25.69 ^{Ea}	23.24 ^{Bb}	21.72 ^{Bc}	21.03 ^{Ea}	18.65 ^{Eb}	15.76 ^{Fc}	7.58 ^{Dc}	8.22 ^{Cb}	9.83 ^{Ca}
	121	29.61 ^{Ja}	21.72 ^{Gb}	19.14 ^{Fc}	8.25 ^{Dc}	9.15 ^{Db}	14.78 ^{Aa}	23.56 ^{Ga}	22.09 ^{Cb}	20.34 ^{Cc}	28.57 ^{Ba}	17.86 ^{Fb}	10.73 ^{Gc}	8.34 ^{Ac}	9.81 ^{Ab}	10.74 ^{Aa}
SEM *		0.025			0.013			0.045			0.037			0.008		

The means in a column with different superscript upper-case letters and in a row with different superscript lower-case letters differ significantly ($p < 0.05$). * Standard error of the mean.

4. Discussion

4.1. Effect of κ-Carrageenan Concentration on Chocolate-Flavoured MPC Dispersions at 20 °C

Under neutral pH conditions (~pH 6.8), an electrostatic interaction occurs between κ-carrageenan and κ-CN at the surface of the casein micelles [24,30]. Specifically, a segment of κ-CN spanning residues 97 to 112 plays a significant role in these interactions, exhibiting a considerable positive charge capable of establishing an electrostatic bond with the negatively charged sulphate groups present in κ-carrageenan [31,32]. κ-Carrageenan is composed of linear polymers that have a backbone structure made up of alternating α-1,4- and β-1,3-linked galactose residues. These polymers also contain different amounts of sulphate half-ester groups, providing a negative charge and playing a role in shaping their properties and functions [33]. Cations such as Ca can also serve as bridges between the carboxyl groups on proteins (e.g., milk protein or proteins from cocoa) and the sulphate groups on carrageenan [34]. As a result, a reduction in soluble minerals such as Ca and Mg were observed alongside the κ-carrageenan concentration [29,35]. Similar interactions involving carboxyl and sulphate groups can also occur in the case of whey proteins at neutral pH [36,37]. This was supported by the FTIR results as alterations observed in α-helical structures along with the potential unfolding of intramolecular β-sheets into more random structures following the addition of κ-carrageenan, indicating their involvement in these interactions. This interplay contributes to the decline in soluble caseins and whey proteins, as observed in SDS PAGE data, a consequence of their interactions with κ-carrageenan as its concentration ascends.

At 20 °C and particularly at lower concentrations, κ-carrageenan serves as an effective stabilizer, preventing the agglomeration of particles in the milk dispersion. The elevation of κ-carrageenan concentration at 20 °C induces the extension of carrageenan molecules, facilitated by the mutual repulsion arising from the abundance of negatively charged half-ester sulphate groups along the polymer chain [38]. Moreover, owing to its hydrophilic nature, the carrageenan molecules become enveloped by a sheath of immobilized water molecules [38]. As the κ-carrageenan concentration increases, the enhanced suspension and stability of milk particles emerges through the formation of a protective barrier enveloping the particles [39]. Additionally, milk dispersions mixed with κ-carrageenan at 20 °C exhibits more heterogeneity due to areas rich in carrageenan and other areas rich in casein micelles [40]. Consequently, these factors contribute to increased particle size. The formation of loosely associated fractal structures following the addition of κ-carrageenan facilitates the immobilization of water within the dispersant. This process augments the effective volume fraction, consequently increasing apparent viscosity [41]. Additionally, the higher solids content contributes to increased viscosity through molecular movements and interfacial film formation [42,43].

4.2. Effect of Heating on Chocolate-Flavoured MPC Dispersions with Varying Levels of κ-Carrageenan

κ-Carrageenan exhibits different conformations depending on the temperature. At elevated temperatures (>50 °C and depending on ion concentration), it exists as a random coil in solution. However, as the temperature decreases, it undergoes a conformational transition from a coil to a helix structure (~37 °C). This helix formation and subsequent aggregation of neighbouring helices promote the gelation process [44,45]. In addition, κ-carrageenan becomes more accessible when it transitions from random coil structures to helices upon binding with positively charged κ-CN [32]. The helical conformation of κ-carrageenan leads to a higher charge density as the sulphate groups come closer together, facilitating electrostatic interactions with casein [46,47]. Furthermore, this helical conformation promotes the sequestration of minerals within these sulphate groups, ultimately leading to reduced levels of minerals in the serum phase. Studies utilizing dynamic light scattering (DLS) have demonstrated that the presence of κ-carrageenan causes an increase in the diameter of casein micelles as the temperature drops below the coil–helix transition temperature [31]. This increase in diameter suggests adsorption and molecular interaction

between κ-carrageenan and casein. Thus, it becomes evident that the incorporation of κ-carrageenan results in a gradual enlargement of particle size (Table 1). However, this trend reverses upon heating. One of the reasons could be the homogenizing effect caused by thermodynamic influences [40]. Another reason could be the micellar disaggregation, in which κ-CN/κ-carrageenan complexes detach from the casein micelles at elevated temperatures, as evidenced by a substantial increase in κ-CN in the soluble phase upon heating (Table 3), resulting in a reduced particle size (Table 1) [48]. On the other hand, in the absence of κ-carrageenan, the interaction of cocoa components, such as polyphenols, with casein proteins can potentially modify their structure and stability of milk proteins, as observed in the results [49]. This interaction, combined with the heating process, further promotes the breakdown of casein micelles into smaller particles, resulting in decreased particle size [49].

The influence of milk proteins was found to be dominant when carrageenan concentration was <0.018% *w*/*w* in milk [33]. Under these conditions, milk proteins interfere with gel formation leading to the domination of protein–carrageenan interactions, reducing the availability of carrageenan molecules in a gelation role. Additionally, a substantial component of protein–protein interactions occurred due to low levels of carrageenan. At high levels of carrageenan (>0.01%), milk proteins have a minimal apparent effect on gelation. Therefore, the association of κ-carrageenan helices resulting in the formation of robust self-supporting gels that entrapped both casein micelles and casein micelle–carrageenan complexes. [33]. Therefore, it is clear that changes in secondary structures were more pronounced at lower κ-carrageenan levels, as interruptions by κ-carrageenan were minimal, as mentioned above, allowing for the increased availability of caseins for potential interactions [50].

In addition to the concentration of κ-carrageenan, the influence of heat can profoundly impact the resulting gelation and apparent viscosity. A higher temperature appears to be required to activate the carrageenan network, as evidenced by the higher consistency factor at 121 °C compared to that at 90 °C depicting a stronger gelation process. The carrageenan molecules may not fully hydrate or transition and interact with proteins and each other, resulting in a less organized and weaker gel structure at 90 °C. In contrast, the higher temperature (121 °C) facilitates the better hydration and transition of the carrageenan molecules, leading to a more organized and tightly bonded gel network. This stronger gel structure can trap more water and milk components, resulting in a higher apparent viscosity of the milk dispersions with κ-carrageenan at 121 °C. Furthermore, as previously mentioned, the detachment of κ-CN/κ-carrageenan complexes at elevated temperatures results in an increase in particle concentration, consequently leading to a rise in consistency factor, and thereby, apparent viscosity. Moreover, it is apparent that the influence of heat is dependent upon the concentration of κ-carrageenan. Specifically, at 0.05% κ-carrageenan concentration, the particle size distribution at both 90 °C and 121 °C underwent a transition from a monomodal to a bimodal distribution. This alteration signifies a relatively larger fraction of particles exceeding the >1 μm range and is indicative of a more pronounced network formation at both temperatures. This observation is substantiated by the heightened consistency factor observed at 0.05% κ-carrageenan concentration in contrast to other dispersions containing κ-carrageenan, thereby resulting in an increased apparent viscosity.

The impact of whey protein denaturation on κ-carrageenan's gelation abilities in milk was studied [6,51], and it was determined that it did not substantially affect κ-carrageenan's gelation. Additionally, the κ-carrageenan present in solution during heat treatment of milk did not have a notable effect on the rate of whey protein denaturation. In fact, the formation of the β-LG/κ-CN complex entails a sulphydryl-disulphide interchange mechanism between the sulphydryl groups on β-LG and the cysteinyl residues located in the hydrophobic domain of κ-CN. Conversely, the interaction between κ-CN and κ-carrageenan is mediated by the glycopeptide region found within the hydrophilic domain of κ-CN and the negatively charged sulphate groups present on the carrageenan molecule [48]. However, the heat-induced denaturation of β-LG may expose more positively charged groups, facilitating electrostatic interactions with the sulphate groups of κ-carrageenan,

ultimately leading to the formation of complexes [52,53]. Hence, heating resulted in a reduction of whey proteins, as evidenced by PAGE analysis, attributed to heat-induced denaturation and subsequent aggregation with other proteins, potentially including κ-carrageenan. On the other hand, α_s-CN, β-CN, and κ-CN levels exhibited a concentration-dependent increase within the soluble phase along with the heating (Table 4).

4.3. Effect of Combined Heat and Shear on Chocolate-Flavoured MPC Dispersions with Varying Levels of κ-Carrageenan

The combined application of heat and shear to milk proteins can lead to a cascade of changes, including denaturation, unfolding, aggregation, the fragmentation of large aggregates, and altered interactions with other components, such as minerals and polysaccharides [10,54,55]. Higher concentrations of κ-carrageenan, in conjunction with shear forces, led to an increase in average particle size and a reduction in non-sedimentable mineral content. This growth in particles in a dispersion due to shear can be attributed to several underlying factors. First, the heightened shear and pressure resulting from rotational diffusion, due to a tumbling motion, facilitated rapid particle association through a combination of hydrophobic and electrostatic interactions [56,57]. Furthermore, the periodic tumbling motion induced by the high shear rates in the fluid flow field may introduce conformational distortions in the molecules, leading to kinked states. These kinked states, in turn, promote the gradual unravelling of molecules and enhance intermolecular hydrodynamic interactions [58,59]. Additionally, molecules brought into proximity via hydrophobic interactions are likely to engage in disulphide bridging during the heating process. This occurs due to the exposure of previously concealed free sulfhydryl groups during the unfolding of molecules [14]. The observed increase in the average particle size at 1000 s^{-1} at higher concentration of κ-carrageenan is substantiated by notable reductions in the levels of κ-CN, αs-CN, and β-CN, as well as both β-LG and α-LA in the serum phase. This reduction strongly suggests their involvement in the overall aggregation, as supported by the SDS-PAGE data. Notably, a more pronounced reduction in β-LG, α-LA, and κ-CN in a concentration-dependent manner implies an increased propensity for aggregation among these specific proteins and with κ-carrageenan under the influence of shear. In addition, as indicated by FTIR data, the gradual decline in intramolecular β-sheets and incline in aggregated β-sheets, when the combined heat and shear was applied to κ-carrageenan-added dispersions, depicts the shear-induced aggregation. The number of β-turns declined as a result of reduced free caseins within the soluble phase. This decrease was most prominent at the highest concentration of κ-carrageenan, owing to the comparatively low availability of caseins due to the entrapment of these in a more robust gel network [60]. The aggregation induced by shear forces, leading to the formation of larger particles, can result in a heightened resistance to flow, consequently prompting an increase in the consistency factor and thereby the apparent viscosity of suspensions (Table 3).

5. Conclusions

The impact of shear on chocolate-flavoured milk dispersions appears primarily dependent on the concentration of κ-carrageenan and heating temperature. At higher carrageenan concentrations, gelation is predominantly driven by interactions among carrageenan molecules themselves, resulting in the entrapment of casein micelles and carrageenan–casein micelle complexes. As a result, the intense destabilization of casein micelles and increased collision rates under shear conditions lead to further aggregation, reducing the presence of both caseins and whey proteins in the soluble phase. Simultaneously, this process increases the average particle size and apparent viscosity within the dispersions.

Given that these processing conditions are commonly encountered in production, it is essential to carefully consider them during food processing to achieve the desired product characteristics while minimizing any potential adverse effects.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12244404/s1. Figure S1: SDS-PAGE analysis of supernatants of MPC dispersions.

Author Contributions: Methodology, A.M., T.H. and J.C.; validation, T.H.; investigation, A.M.; resources, J.C. and T.V.; data curation, A.M.; writing—original draft, A.M.; writing—review and editing, A.M., T.H., J.C. and T.V.; supervision, J.C. and T.V.; project administration, T.V.; funding acquisition, T.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Victoria University Research Training Stipend Scholarship (grant number—5110477).

Data Availability Statement: Data is contained within the article or supplementary material.

Conflicts of Interest: Thom Huppertz is employed by the company FrieslandCampina. The research was conducted independently, without any commercial or financial affiliations with the aforementioned company, thereby mitigating the potential for conflict of interest.

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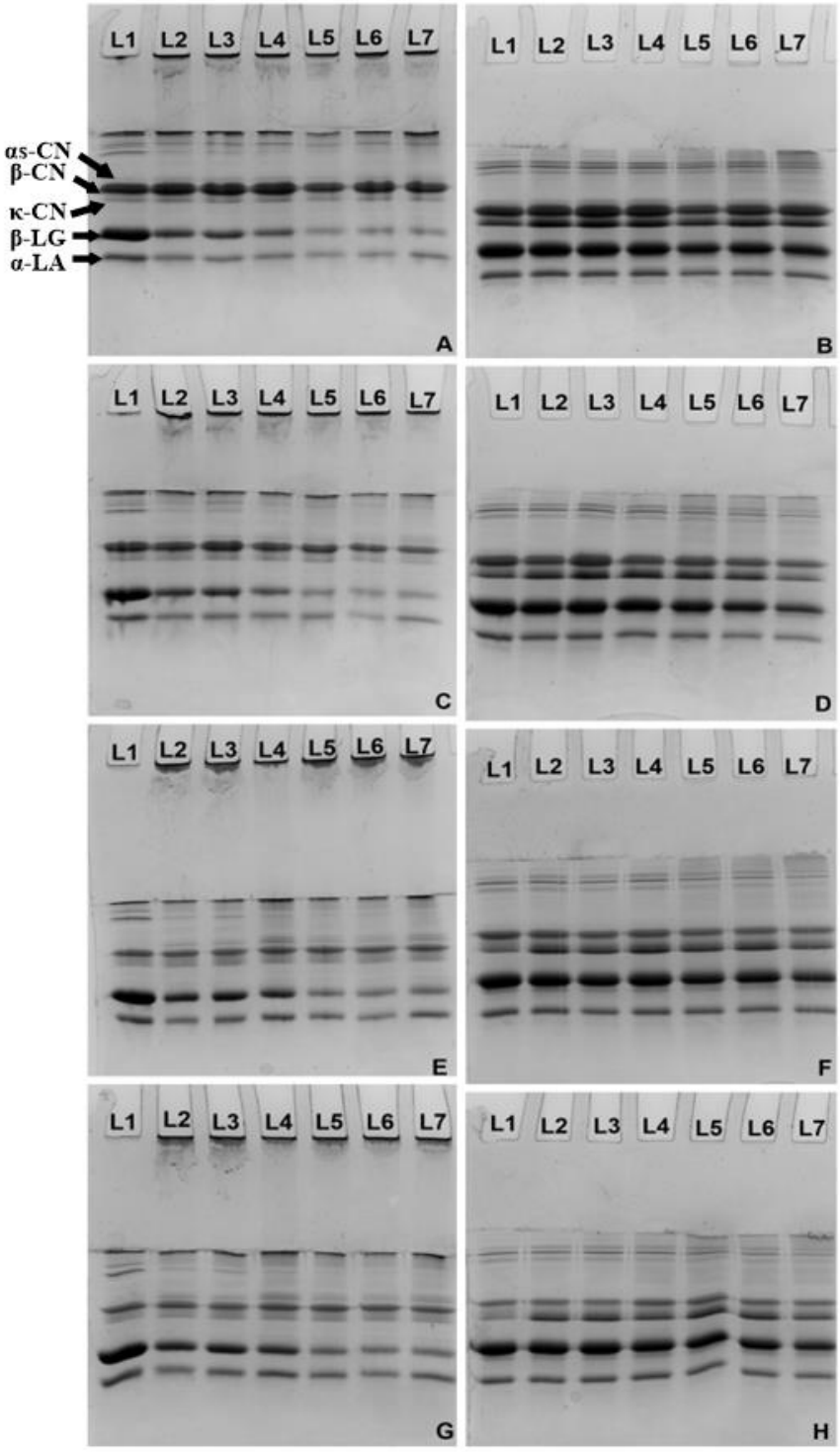
Figure S1: SDS-PAGE analysis of supernatants of MPC dispersions non-reducing (A) and reducing (B) of 0% κ-carrageenan (1st raw), non-reducing (C) and reducing (D) of 0.01% κ-carrageenan (2nd raw) , non-reducing (E) and reducing (F) of 0.03% κ-carrageenan (3rd raw) and non-reducing (G) and reducing (H) of 0.05% κ-carrageenan (4th raw) from left to right. Lane sequence: control (25-0)/90-0/90-100/90-1000/121-0/-121-100/121-1000 from left to right.



CHAPTER 7. Conclusions and Future Perspectives

7.1 Conclusions

In recent years, the growing interest in improving health and nutrition has significantly increased the demand for high-protein dairy products. These products cater to the nutritional needs of a diverse consumer base, from athletes seeking muscle repair and growth to elderly individuals aiming for maintenance and health of their muscle mass. MPCs play a pivotal role in the development of dairy products that boast higher protein content, improved texture, and enhanced shelf-life. The critical role of these products in modern diets underscores the imperative for continuous innovation and optimization in their production processes. A thorough understanding of how processing conditions such as heating and shearing impact the stability and functional properties of MPCs is crucial. The investigation into the effects of shearing and heating on the functionality of MPCs, alongside the influence of protein levels, pH adjustments, CSS, and the incorporation of κ-carrageenan, reveals critical insights into protein behaviour during processing. This body of work underscores the complexity of protein interactions under various conditions and the potential to optimize food processing techniques for enhanced product stability and quality.

Heat stability of MPCs was significantly influenced by protein concentration under the impact of shear as observed within the first study. In the comparative analysis of MPC suspensions containing 4% and 8% protein concentrations, it has been observed that the elevated shear rates up to 1500 s^{-1} substantially compromised the heat stability of suspensions at the higher protein concentration. An augmentation in protein concentration to 8% significantly intensifies protein-protein interactions, rendering the proteins more susceptible to aggregation under conditions of elevated heating up to 121 °C. At increased shear rates, the cohesive stabilising forces important in maintaining the protein helical conformation, such as intramolecular hydrogen bonds, were overridden by hydrodynamic drag forces, given rise to molecular unfolding. This unfolding process reveals reactive sites that were previously occluded, facilitating the formation of complexes between whey proteins and casein micelles, which in turn facilitated further aggregation at

heightened temperatures, thereby diminishing heat stability. Consequently, given these parameters, it was deduced that a lower protein content of 4% and reduced temperature settings are more conducive to achieving optimal heat stability in the context of high shear conditions for reconstituted milk products.

This prominent shear-induced aggregation at 8% protein concentration was further modulated by the physicochemical environment, by adjusting pH within the MPC suspensions which can either diminish or enhance the destabilizing effects of shear and temperature. For instance, at pH 7.5, shear facilitated the dissociation of micelles, through shear-induced deformation and destabilization. In contrast, at pH values of 6.1 and 6.4, the application of shear appeared to notably alter the electrostatic environment and the extent of association between whey proteins and micelles. Specifically, at pH of 6.1, shear was observed to facilitate heat induced protein aggregation, as demonstrated by an increase in particle size and a marked decrease in the solubility of both whey proteins and caseins. Conversely, at a pH of 6.4, shear forces led to pronounced fragmentation, a phenomenon attributed to elevated electrostatic repulsions and diminished protein-protein interactions.

The influence of shear was primarily dictated by the calcium-binding ability of CSS, DSHP and TSC. The pronounced impact of shear on casein micelles within dispersions containing DSHP evidenced a reduced calcium affinity. This reduced affinity attenuates micellar disruption, improve surface area for molecular encounters, and intensifies hydrodynamic fluid dynamics consequent to the precipitation of calcium phosphate. This leads to decrease in electrostatic repulsions, thereby facilitating the micellar interactions. On the other hand, the elevated calcium affinity associated with TSC resulted in more profound disruption of micelles, releasing caseins and causing protein aggregation as a consequence of shear forces.

The impact of shear forces on chocolate-flavoured milk suspensions primarily depends on the concentration of κ-carrageenan and the applied heating temperature. At higher concentrations of κ-carrageenan, gelation was predominantly arisen from intermolecular interactions among carrageenan molecules themselves, leading to the entrapment of both casein micelles and carrageenan-casein micelle complexes. Consequently, this

phenomenon induces pronounced destabilization of casein micelles and augments collision rates under shear conditions, thereby facilitating further aggregation and a subsequent reduction in the solubility of both caseins and whey proteins. This was primarily evidenced through increased average particle size and apparent viscosity within the MPC suspensions. Given the prevalence of such processing conditions in food processing operations, meticulous consideration of these factors is imperative to achieve the desired product attributes while mitigating potential deleterious effects.

These findings have profound implications for the dairy and food processing industries. By understanding the intricate effects of shear, temperature, and physico-chemical conditions on protein stability, manufacturers can optimize processing parameters to improve product quality, consistency, and functionality. This could involve adjusting protein concentrations and pH, carefully selecting additives like CSS or κ-carrageenan, and controlling processing conditions such as shear rates and temperatures to minimize undesirable protein interactions. In conclusion, current study highlights the critical role of processing conditions in determining the heat stability and functional properties of milk proteins in food products. By leveraging these insights, the food industry can enhance product development and innovation, ensuring that dairy products meet the evolving demands of consumers for quality, stability, and nutritional value. Further research in this domain will undoubtedly continue to shed light on the intricate mechanisms governing protein behaviour, paving the way for more refined and efficient food processing techniques.

7.2 Future perspectives

The findings and insights derived from the current study on the impact of processing conditions on MPCs in the production of high-protein dairy beverages pave the way for several avenues of future research and development within the dairy industry. The evolving landscape of consumer preferences towards healthier, protein-rich diets presents a unique opportunity for the dairy sector to innovate and refine the production of high-protein beverages.

Future perspectives should focus on expanding the scope of research to include a wider variety of MPCs and dairy formulations, considering the inherent variability in protein and mineral content. This would enable a more comprehensive understanding of how different MPCs respond to various processing conditions, facilitating the tailoring of processing techniques to optimize product quality and functionality. One key area for future investigation is the integration of novel proteins or processing methods guided by the observed impacts of shear and temperature. For example, based on the findings in this study regarding the destabilizing effects of shear at higher protein concentrations, future research could focus on developing innovative processing techniques that alleviate these effects while maintaining product quality. This could involve novel approaches to controlling shear forces during processing or exploring alternative protein sources with enhanced stability under shear conditions.

Furthermore, further investigation into the integration of novel ingredients that can complement MPCs to enhance the texture, flavour, and nutritional profile of dairy beverages under such processing conditions would be an important aspect. For example, exploring the use of plant-based proteins, probiotics, and bioactive peptides holds promise for developing next-generation dairy products tailored to the preferences of health-conscious consumers, while maintaining the sensory characteristics that define high-quality dairy products.

Additionally, it is relevant to delve deeper into how changes in functionality resulting from different processing techniques may affect the human immune system, potentially influencing immunogenic and antigenic responses. This would provide valuable insights into the potential risks and benefits associated with various processing methods in relation to human consumption.

Finally, sustainability considerations should be integrated into future research efforts, focusing on reducing the environmental impact of dairy processing. This includes exploring energy-efficient processing methods, waste reduction strategies, and sustainable sourcing of ingredients. Moreover, understanding these aspects would be useful in process improvements by minimizing detrimental effects such as scaling and protein precipitation on surfaces in production lines. By reducing scaling and protein

precipitation, less cleaning chemicals and water are required, leading to decreased resource consumption and waste generation. This not only improves operational efficiency but also contributes to environmental sustainability.

In conclusion, the path forward for the dairy industry is one of innovation and adaptation, driven by scientific research and consumer demand. By building on the foundational work presented in this thesis, the industry can continue to advance the development of high-quality, protein-rich dairy products that meet the needs of today's and tomorrow's consumers.