



# 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<sup>-1</sup>) 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.

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## 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  $\alpha_s$ - and  $\beta$ -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, Puvaenthiran, 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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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 (Mediawaththe, Bogahawaththa, Grewal, Chandrapala, & Vasiljevic, 2018a; Mediawaththe, 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 ( $1000\text{ s}^{-1}$ ) 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 ( $90\text{ }^{\circ}\text{C}/5\text{ min}$  and  $121\text{ }^{\circ}\text{C}/2.6\text{ min}$ ) and shear rates (100, 1000, or  $1500\text{ s}^{-1}$ ). 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  $-20\text{ }^{\circ}\text{C}$ . 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  $50\text{ }^{\circ}\text{C}$  for complete solubilisation of powder and kept at  $4\text{ }^{\circ}\text{C}$  overnight for further hydration (Liyanaarachchi & Vasiljevic, 2018). The following day, the samples were equilibrated at  $25\text{ }^{\circ}\text{C}$  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  $25\text{ }^{\circ}\text{C}$ , 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 drop-wise 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 ( $90\text{ }^{\circ}\text{C}$  for 5 min and  $121\text{ }^{\circ}\text{C}$  for 2.6 min) at a shear rate of 100, 1000 or  $1500\text{ s}^{-1}$  in a pressure cell (CC25/PR-150) of a rheometer (Physica MCR 301 series, Anton Paar GmbH, Ostfildern-Schornhausen, 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  $5\text{ }^{\circ}\text{C min}^{-1}$  to the required temperature and held there for the required time and cooled at a rate of  $5\text{ }^{\circ}\text{C min}^{-1}$ . 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  $100,000\times g$  for 1 h at  $20\text{ }^{\circ}\text{C}$  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  $15\text{ }^{\circ}\text{C}$  with a SEPA CF membrane module and polyethersulfone (PES) membrane ( $190\times 140\text{ mm}$ ) 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  $\beta$ -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<sub>3</sub> 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<sup>-1</sup>.<sup>a</sup>

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

<sup>a</sup> 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  $100\text{ s}^{-1}$  when compared with the control at  $25\text{ }^{\circ}\text{C}$ . This decrease was observed at both  $90\text{ }^{\circ}\text{C}$  and  $121\text{ }^{\circ}\text{C}$ . These levels gradually increased, reaching their peak at a shear rate of  $1500\text{ s}^{-1}$ , and ultimately reaching similar levels to those of the control at  $25\text{ }^{\circ}\text{C}$  (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  $90\text{ }^{\circ}\text{C}$  and  $121\text{ }^{\circ}\text{C}$ , at all CSS levels, with a more pronounced effect at  $121\text{ }^{\circ}\text{C}$  (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  $121\text{ }^{\circ}\text{C}$ ) (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  $\kappa$ -CN,  $\alpha$ -CN, and  $\beta$ -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  $\beta$ -LG and  $\alpha$ -LA declined in the serum phase during heating, although the  $\kappa$ -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  $\kappa$ -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  $121\text{ }^{\circ}\text{C}$ , 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  $121\text{ }^{\circ}\text{C}$ ) (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  $120\text{ }^{\circ}\text{C}$  and shearing at  $100$ ,  $1000$  or  $1500\text{ s}^{-1}$ .<sup>a</sup>

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

<sup>a</sup> 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<sup>-1</sup> 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<sup>-1</sup> likely indicates dissociated casein particles. At 1500 s<sup>-1</sup>, 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  $\beta$ -lactoglobulin and  $\alpha$ -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  $\alpha$ <sub>S</sub>-,  $\beta$ -, and  $\kappa$ -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  $\beta$ -LG and  $\alpha$ -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  $\alpha$ <sub>S</sub>-,  $\beta$ -, and  $\kappa$ -CNs, with a relatively greater rise in  $\kappa$ -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 <sup>-1</sup> )	0 mm			10 mm			20 mm			30 mm		
		$\alpha$ <sub>S</sub> -CN	$\beta$ -CN	$\kappa$ -CN	$\alpha$ <sub>S</sub> -CN	$\beta$ -CN	$\kappa$ -CN	$\alpha$ <sub>S</sub> -CN	$\beta$ -CN	$\kappa$ -CN	$\alpha$ <sub>S</sub> -CN	$\beta$ -CN	$\kappa$ -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									
121	1500	2.0	2.6	33.1									
	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
121	1500				19.8	15.3	63.1	20.2	49.9	73.5	60.4	64.5	76.8
	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
121	1500				17.8	27.8	58.1	38.7	38.1	61.3	55.3	60.3	78.8
	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 <sup>-1</sup> )	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					
121		1500	53.1	65.2					
	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<sup>-1</sup> and 1500 s<sup>-1</sup> (Table 3; Supplementary material Fig. S3). However, the heat load in combination with shear released less α<sub>5</sub>- and β-CN 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<sub>5</sub>-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<sup>-1</sup> 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<sup>-1</sup>. In addition, all the α<sub>5</sub>-, 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<sub>5</sub>-, 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; Mediawaththe 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 (Stevenson, 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 (Mediawaththe 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  $\text{HPO}_4^{2-}$  ( $600 \text{ M}^{-1}$ ), as opposed to citrate's higher affinity ( $10^5 \text{ M}^{-1}$ ) for calcium (Kapoor, Metzger, Biswas & Muthukumarappan, 2007; Walstra & Jenness, 1984). Addition of TSC at  $25^\circ\text{C}$  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  $30 \text{ mM}$  TSC and at  $121^\circ\text{C}$  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  $\kappa$ -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  $\kappa$ -CN,  $\beta$ -LG would also have been associated with  $\alpha_{\text{S2}}$ -CN within the  $\kappa$ -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  $\beta$ -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  $\beta$ -CN and  $\alpha_{\text{S}}$ -CN was observed at  $20 \text{ mM}$ , and further increase in concentration up to  $30 \text{ mM}$  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  $4 \text{ nm}$  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  $\alpha_{\text{S2}}$ -CN >  $\alpha_{\text{S1}}$ -CN >  $\beta$ -CN >  $\kappa$ -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  $100 \text{ s}^{-1}$  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  $\kappa$ -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>.

## References

- Agarwal, S., Beausire, R. L., Patel, S., & Patel, H. (2015). Innovative uses of milk protein concentrates in product development. *Journal of Food Science*, 80, A23–A29.
- Anema, S. G. (2023). The turbidity of heated milk in relation to particle size and protein distributions between the casein micelles and the serum phase. *International Dairy Journal*, 147. Article 105771.
- Anema, S. G., Lee, S. K., & Klostermeyer, H. (2022). Reconstituted skim milk: Effect of re-dilution after heating concentrated milk at different pH and temperatures. *International Dairy Journal*, 131. Article 105368.
- Augustin, M.-A., & Clarke, P. T. (1990). Effects of added salts on the heat stability of recombinant concentrated milk. *Journal of Dairy Research*, 57, 213–226.
- Bijl, E., Huppertz, T., van Valenberg, H., & Holt, C. (2019). A quantitative model of the bovine casein micelle: Ion equilibria and calcium phosphate sequestration by individual caseins in bovine milk. *European Biophysics Journal*, 48, 45–59.
- Bogahawaththa, D., Buckow, R., Chandrapala, J., & Vasiljevic, T. (2018). Comparison between thermal pasteurization and high pressure processing of bovine skim milk in relation to denaturation and immunogenicity of native milk proteins. *Innovative Food Science & Emerging Technologies*, 47, 301–308.
- Bogahawaththa, D., & Vasiljevic, T. (2020). Shearing accelerates denaturation of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin in skim milk during heating. *International Dairy Journal*, 105. Article 104674.
- Broyard, C., & Gaucheron, F. (2015). Modifications of structures and functions of caseins: A scientific and technological challenge. *Dairy Science & Technology*, 95, 831–862.
- Carr, A., & Golding, M. (2016). Functional milk proteins production and utilization: Casein-based ingredients. In P. L. H. McSweeney, & P. F. Fox (Eds.), *Advanced dairy chemistry* (pp. 35–66). New York, NY, USA: Springer.
- Chandrapala, J., Duke, M. C., Gray, S. R., Zisu, B., Weeks, M., Palmer, M., et al. (2015). Properties of acid whey as a function of pH and temperature. *Journal of Dairy Science*, 98, 4352–4363.
- Corredig, M., Nair, P. K., Li, Y., Eshpari, H., & Zhao, Z. (2019). Invited review: Understanding the behavior of caseins in milk concentrates. *Journal of Dairy Science*, 102, 4772–4782.
- Cross, K. J., Huq, N. L., Palamara, J. E., Perich, J. W., & Reynolds, E. C. (2005). Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes. *Journal of Biological Chemistry*, 280, 15362–15369.
- Daniloski, D., McCarthy, N. A., & Vasiljevic, T. (2022). Impact of heating on the properties of A1/A1, A1/A2, and A2/A2  $\beta$ -casein milk phenotypes. *Food Hydrocolloids*, 128. Article 107604.
- Davies, D. T., & Law, A. J. R. (1977). An improved method for the quantitative fractionation of casein mixtures using ion-exchange chromatography. *Journal of Dairy Research*, 44, 213–221.
- De Kort, E., Minor, M., Snoeren, T., Van Hooijdonk, T., & Van Der Linden, E. (2009). Calcium-binding capacity of organic and inorganic ortho- and polyphosphates. *Dairy Science & Technology*, 89, 283–299.
- de Kort, E. J. (2012). Influence of calcium chelators on concentrated micellar casein solutions: From micellar structure to viscosity and heat stability (*PhD thesis*). Wageningen, The Netherlands: Wageningen Agricultural University.
- Di Stasio, E., & De Cristofaro, R. (2010). The effect of shear stress on protein conformation: Physical forces operating on biochemical systems: The case of von Willebrand factor. *Biophysical Chemistry*, 153, 1–8.
- Fang, Y., Selomulya, C., Ainsworth, S., Palmer, M., & Chen, X. D. (2011). On quantifying the dissolution behaviour of milk protein concentrate. *Food Hydrocolloids*, 25, 503–510.
- García, A., Altig, A., & Huppertz, T. (2023). Disruption of casein micelles by calcium sequestering salts: From observations to mechanistic insights. *International Dairy Journal*, 142. Article 105638.
- Gaucher, I., Piot, M., Beaucher, E., & Gaucheron, F. (2007). Physico-chemical characterization of phosphate-added skim milk. *International Dairy Journal*, 17, 1375–1383.
- Griffin, M. C. A., & Griffin, W. G. (1985). A simple turbidimetric method for the determination of the refractive index of large colloidal particles applied to casein micelles. *Journal of Colloid and Interface Science*, 104, 409–415.
- Havea, P. (2006). Protein interactions in milk protein concentrate powders. *International Dairy Journal*, 16, 415–422.
- Huppertz, T., Fox, P., & Kelly, A. (2018). The caseins: Structure, stability, and functionality. In R. Y. Yada (Ed.), *Proteins in food processing* (pp. 49–92). London, UK: Woodhead Publishing.
- Kapoor, R., Metzger, L. E., Biswas, A. C., & Muthukumarappan, K. (2007). Effect of natural cheese characteristics on process cheese properties. *Journal of Dairy Science*, 90, 1625–1634.
- Liyanaarachchi, W., Ramchandran, L., & Vasiljevic, T. (2015). Controlling heat induced aggregation of whey proteins by casein inclusion in concentrated protein dispersions. *International Dairy Journal*, 44, 21–30.
- Liyanaarachchi, W., & Vasiljevic, T. (2018). Caseins and their interactions that modify heat aggregation of whey proteins in commercial dairy mixtures. *International Dairy Journal*, 83, 43–51.
- Lucey, J., & Horne, D. (2009). Milk salts: Technological significance. In P. L. H. McSweeney, & P. F. Fox (Eds.), *Advanced dairy chemistry* (pp. 351–389). New York, NY, USA: Springer.
- Markoska, T., Huppertz, T., Grewal, M. K., & Vasiljevic, T. (2019). FTIR analysis of physicochemical changes in raw skim milk upon concentration. *Lebensmittel-Wissenschaft und -Technologie*, 102, 64–70.
- Martinie, G. D., & Schilt, A. A. (1976). Wet oxidation efficiencies of perchloric acid mixtures for various organic substances and the identities of residual matter. *Analytical Chemistry*, 48, 70–74.
- Mediawaththe, A., Bogahawaththa, D., Grewal, M. K., Chandrapala, J., & Vasiljevic, T. (2018a). Structural changes of native milk proteins subjected to controlled shearing and heating. *Food Research International*, 114, 151–158.
- Mediawaththe, A., Chandrapala, J., & Vasiljevic, T. (2018b). Shear-induced behaviour of native milk proteins heated at temperatures above 80 °C. *International Dairy Journal*, 77, 29–37.
- Mejares, C. T., Chandrapala, J., & Huppertz, T. (2023). Influence of calcium-sequestering salts on heat-induced changes in blends of skimmed Buffalo and bovine milk. *Foods*, 12. Article 2260.
- Mizuno, R., & Lucey, J. (2005). Effects of emulsifying salts on the turbidity and calcium-phosphate–protein interactions in casein micelles. *Journal of Dairy Science*, 88, 3070–3078.
- Mizuno, R., & Lucey, J. A. (2007). Properties of milk protein gels formed by phosphates. *Journal of Dairy Science*, 90, 4524–4531.
- Rajan, R., Ahmed, S., Sharma, N., Kumar, N., Debas, A., & Matsumura, K. (2021). Review of the current state of protein aggregation inhibition from a materials chemistry perspective: Special focus on polymeric materials. *Materials Advances*, 2, 1139–1176.
- Ramchandran, L., Luo, X., & Vasiljevic, T. (2017). Effect of chelators on functionality of milk protein concentrates obtained by ultrafiltration at a constant pH and temperature. *Journal of Dairy Research*, 84, 471–478.
- Ranadheera, C., Liyanaarachchi, W., Dissanayake, M., Chandrapala, J., Huppertz, T., & Vasiljevic, T. (2019). Impact of shear and pH on properties of casein micelles in milk protein concentrate. *Lebensmittel-Wissenschaft und -Technologie*, 108, 370–376.
- Renhe, I. R. T., Indris, L. M., & Corredig, M. (2018). Effect of calcium chelators on heat stability and heat-induced changes of milk microfiltered concentrates. *International Dairy Journal*, 82, 4–10.
- Schokker, E. P., & Dalgleish, D. G. (2000). Orthokinetic flocculation of caseinate-stabilized emulsions: Influence of calcium concentration, shear rate, and protein content. *Journal of Agricultural and Food Chemistry*, 48, 198–203.
- Singh, H. (2004). Heat stability of milk. *International Journal of Dairy Technology*, 57, 111–119.
- Stevenson, A. J., Donald, A. M., & Gladden, L. F. (1994). Thermal aggregation of whey protein concentrates under fluid shear conditions. In A. T. Andrews, & J. R. Varley (Eds.), *Biochemistry of milk products* (p. 133). London, UK: Woodhead Publishing. —133.
- Swaigood, H. E. (1992). Chemistry of the caseins. In P. F. Fox, & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry* (Vol. 1, pp. 63–110). Boston, MA, USA: Proteins. Springer.
- Tessier, H., & Rose, D. (1958). Calcium ion concentration in milk. *Journal of Dairy Science*, 41, 351–359.
- Tsioulpas, A., Koliandris, A., Grandison, A. S., & Lewis, M. J. (2010). Effects of stabiliser addition and in-container sterilisation on selected properties of milk related to casein micelle stability. *Food Chemistry*, 122, 1027–1034.
- Udabage, P., Puvanenthiran, A., Yoo, J. A., Versteeg, C., & Augustin, M. A. (2012). Modified water solubility of milk protein concentrate powders through the application of static high pressure treatment. *Journal of Dairy Research*, 79, 76–83.
- Vujicic, I., DeMan, J., & Woodrow, I. (1968). Interaction of polyphosphates and citrate with skimmilk proteins. *Canadian Institute of Food Technology Journal*, 1, 17–21.
- Walstra, P. (2001). Effects of agitation on proteins. In E. Dickinson, & R. Miller (Eds.), *Food colloids: Fundamentals of formulation* (pp. 245–254). Cambridge, UK: Royal Society of Chemistry.
- Walstra, P., & Jenness, R. (1984). *Dairy chemistry & physics*. New York, NY, USA: John Wiley & Sons.