International Dairy Journal 142 (2023) 105638

Contents lists available at ScienceDirect

International Dairy Journal

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

Disruption of casein micelles by calcium sequestering salts: From observations to mechanistic insights

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ARTICLE INFO

Article history:
Received 13 October 2022
Received in revised form
21 February 2023
Accepted 24 February 2023
Available online 15 March 2023

ABSTRACT

In this study, the disruption of casein micelles through the addition of the calcium sequestering salts trisodium citrate (TSC) and sodium hexametaphosphate (NaHMP) was investigated in micellar casein isolate (MCI) suspensions containing 9% casein. TSC- and NaHMP-induced disruption of casein micelles was apparent from decreases in turbidity and increases in non-sedimentable casein and calcium. Changes in particle size were found not to correlate to micellar disruption, presumably due to residual particles dominating intensity-based particle size distributions. Decreases in Ca-ion activity confirmed the Ca-sequestering activity of TSC and NaHMP. However, while both Ca-sequestering salts disrupted casein micelles, their mode of action appears very different, but this could only be distinguished when also considering changes in non-permeable Ca. Whereas TSC acts through forming soluble Ca-citrate complexes and solubilises inorganic phosphate, NaHMP mainly appears to act through peptisation reactions, wherein micellar calcium phosphate (MCP) nanoclusters are disrupted but not solubilised.

1. Introduction

Around 80% of the total protein in bovine milk is constituted by caseins (CN), and approximately 20% are whey proteins. They are 4 main caseins in milk: α_{S1}-CN (40%), β-CN (35%), κ-CN (15%) and α_{S2}-CN (10%) (Broyard & Gaucheron, 2015). The casein proteins form casein micelles via protein−protein interactions and also due to the bridging effect of the micellar calcium phosphate (MCP) nanoclusters, where α_{S1}-CN, α_{S2}-CN and β-CN link with MCP nanoclusters via their phosphoserine centres (Dalgleish & Corredig, 2012). Casein micelles are colloidally stabilised by steric and electrostatic repulsion between the κ-CN layers on the surface of the micelles (Walstra, 1990). In normal conditions, the casein micelles are very stable, but their structure can be compromised when their physicochemical conditions change, which is very common during technological operations: changes in pH, severe heat treatments, and changes in the ionic environment, such as the addition of cations or addition of calcium sequestering agents (Gaucheron, 2005).

The addition of calcium sequestering salts to milk systems is practiced in the dairy industry with different purposes. For example, they can be added to improve heat stability and prevent sedimentation in UHT-treated milk (Anema, 2019) or to control the physical properties of processed cheese, such as meltability or texture (Kapoor & Metzger, 2008). Calcium sequestration alters the mineral equilibria, i.e., the distribution of calcium between the colloidal and soluble phase in dairy systems (Power, Fenelon, O'Mahony, & McCarthy, 2019); they act by sequestering the free calcium ions present in dairy systems, and depending on their structure, they can also interact with calcium from MCP (Mizuno & Lucey, 2005), thereby compromising the micelle integrity.

However, the extent to which calcium sequestrants affect the micelle structure differs (De Kort, Minor, Snoeren, Van Hooijdonk, & Van der Linden, 2009), as does their capacity to interact with calcium ions and proteins from the micelle (Mizuno & Lucey, 2007). In addition to effects on performance in products, the mechanistic aspects of the action of calcium sequestering salts on casein micelles has also been studied, and various mechanistic insights have been presented. Lin, Leong, Dewan, Bloomfield, and Morr (1972) reported that EDTA causes partial dissociation of casein micelles as a consequence of calcium depletion from micelles and the

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https://doi.org/10.1016/j.idairyj.2023.105638

release of β-CN and κ-CN. Mizuno and Lucey (2007) indicated that trisodium citrate (TSC) chelates calcium from MCP forming soluble complexes and promotes an increase in the dispersion of casein micelles. Other studies based on polyphosphates such as sodium hexametaphosphate (NaHMP), suggested that NaHMP has the ability of binding the serum phase calcium and also of forming a NaHMP–calcium complex associate with the casein micelles (Anema, 2019). Other authors indicated that NaHMP can bind caseins through their positively charged amino acids (De Kort, Minor, Snoeren, Van Hooijdonk, & Van der Linden, 2012; Mizuno & Lucey, 2005, 2007).

Thus, despite of the extensive research carried out over the years, it is not clear how NaHMP binds CN, although it is generally accepted that the polyphosphate induces (partial) micelle disruption. The aim of this study was to better understand how the calcium sequestrants induce the micelle disruption as well as if NaHMP binds caseins. For that, TSC and NaHMP were used as disruptive agents. A micellar casein isolate (9% CN content) was prepared at three different pH values: 6.5, 6.7, and 6.9 with different amounts of TSC or NaHMP. Calcium-ion activity, turbidity, particle size, protein and mineral equilibria, and viscosity were determined. This work provides a better understanding of the processes where the complexing salts are involved.

2. Materials and methods

2.1. Sample preparation

Micellar casein isolate (MCI) retentate (total solids 18%, total protein 16%, casein content 14.5%, lactose content <0.5%, fat content <0.5%) was obtained from FrieslandCampina (Lochem, The Netherlands) and stored at 4 °C until use. The MCI was diluted to 9% of casein with demineralised water at room temperature. Hereafter, different amounts of stock solution of NaHMP or TSC (in the form of trisodium citrate dihydrate; Sigma-Aldrich GmbH, Germany) were added at concentrations of 0–50 mM (corresponding to 0 to 300 mEq L^{-1} for NaHMP and 0 to 150 mEq L^{-1} for TSC; Table 1). The pH of the samples was adjusted to 6.5, 6.7 or 6.9 (±0.05) using 0.1 M HCl or NaOH, and 0.02% sodium azide (Sigma-Aldrich GmbH, Germany) was added to prevent microbial growth.

2.2. Calcium ion activity

The calcium ion activity was measured using a calcium ion-selective electrode (Sension +9660C; Hach, Loveland, CO, USA) as described by Crowley et al. (2014).

2.3. Calcium and protein distributions

To separate the soluble fraction and the sedimentable material of the solutions, the samples were first diluted 3-fold with demineralised water and subsequently centrifuged at 100,000 × *g* for 1 h at 20 °C. Pellet and supernatant were separated by decanting and weighed. The supernatant was placed in an Amicon Ultra-15 centrifugal tube with a 10 kDa molecular mass cut-off membrane (Amicon, Inc., Beverly, MA, USA) and were centrifuged at 4000 × *g* for 20 min at 20 °C.

The content of Ca in the whole samples, in the ultracentrifugal supernatants and in the 10 kDa permeate were determined by ICP-AES as described by Cruijsen, Poitevin, and Brunelle (2019). Concentrations of Ca in the non-sedimentable fraction and in the non-permeable fraction are expressed as a % of total Ca.

The protein composition of the whole samples and the ultracentrifugal supernatants were determined by RP-HPLC using a method adapted from Visser, Slangen, and Rollema (1991). Values for individual caseins in the ultracentrifugal are expressed as a % of the concentration in the whole sample.

2.4. Turbidity and particle size

The absorbance of the samples was measured at 600 nm at room temperature with a spectrophotometer (Eppendorf, Germany) following 10 or 100-fold dilution with demineralised water to be within the linear range of the spectrophotometer. Reported values are corrected for the dilution.

Particle size of samples diluted 100-fold with demineralised water was analysed in triplicate by dynamic light scattering using a ZetasizerNano (Malvern Instruments, Malvern, UK) at 25 °C at a scattering angle of 173°. Values are expressed as a z-average hydrodynamic diameter (in nm).

2.5. Viscosity

The viscosity of the undiluted samples was determined at 20 °C with a Discovery hybrid rheometer HR-2 (TA Instruments, New Castle, DE, USA) using cup and bob geometry. Samples were conditioned at 20 °C for 120 s, followed by 0.1 s^{-1} for 60 s 0.1 to 1000 s^{-1} over 300 s, 1000 to 0.1 over 300 s, and finally at 0.1 s^{-1} for 60 s. Data points were collected each 5 s. Viscosity results presented are at a shear rate of 100 s^{-1} in the upward curve.

3. Results and discussion

While casein micelles show remarkable stability on, e.g., boiling or freezing of milk, their structural integrity may be compromised. Principally, structural integrity of casein micelles can be disrupted through two main routes, i.e., through disturbance of casein–casein interaction, or through destabilisation of the calcium phosphate nanoclusters which cement the primary casein particles (Huppertz et al., 2017) into a micellar structure and/or the interaction of phosphoserine residues on the caseins therewith (Huppertz & Gazi, 2022; Huppertz, Fox, & Kelly, 2018). Casein–casein interactions can be disrupted by various means. Increasing net-negative charge on the caseins, through, e.g., increases in pH (Vaia, Smiddy, Kelly, & Huppertz, 2006) or conversion of glutamine residues to glutamic acid residues via enzymatic deamidation (Miwa, Yokoyama, Wakabayashi, & Nio, 2010) leads to micellar disruption via disrupted protein–protein interactions. Furthermore, the addition of chaotropic agents, e.g., urea (McGann & Fox, 1974; Smiddy, Martin, Kelly, De Kruif, & Huppertz, 2006) or surfactants, e.g., SDS (Lefebvre-Cases, Gastaldi, & Tarodo de la Fuente, 1998) disrupts

Table 1
Concentrations of calcium sequestering salts (CSS; mmol kg^{-1}) trisodium citrate (TSC; mEq L^{-1}) and sodium hexametaphosphate (NaHMP; mEq L^{-1}) used.

CCS type	Charge	CSS concentration									
		0 mmol kg^{-1}	2 mmol kg^{-1}	4 mmol kg^{-1}	6 mmol kg^{-1}	8 mmol kg^{-1}	10 mmol kg^{-1}	20 mmol kg^{-1}	30 mmol kg^{-1}	40 mmol kg^{-1}	50 mmol kg^{-1}
TSC	−3	0	6	12	18	24	30	60	90	120	150
NaHMP	−6	0	12	24	36	48	60	120	180	240	300

casein–casein interactions and leads to disruption of casein micelles.

Disruption of casein micelles via disruption of calcium phosphate nanoclusters can also be achieved by several means. The addition of calcium sequestrants, which is discussed in further detail below, is a main route for casein micelle disruption. Reducing pH also leads to solubilisation of MCP (Dalgleish & Law, 1989; Le Graët & Gaucheron, 1999; Mekmene, Le Graët, & Gaucheron, 2010) and can therefore lead to micellar disruption, although this is typically countered by enhanced casein–casein interactions at lower pH (Horne, 2008). Disruption of casein micelles at elevated pressure is also governed through solubilisation of MCP. Huppertz and De Kruif (2006), estimated that at ~400 MPa, all MCP in milk had solubilised. At this pressure, extensive micelle disruption is observed through light scattering measurements at high pressure (Huppertz, Kelly, & De Kruif, 2006; Orlien, Knudsen, Colon, & Skibsted, 2006). Finally, treatment of casein micelles with cation exchange resins, which replace calcium for sodium or potassium ions, can also lead to micellar disruption (Xu et al., 2016).

In relation to the disruption of casein micelles via the action of calcium sequestering salts, different salts can be considered, e.g., citrates, orthophosphates, diphosphates, polyphosphates or EDTA, the mode of action of which can differ (Vujicic, DeMan, & Woodrow, 1968). All can complex with free calcium ions in solution, leading to a reduction in calcium ion activity, as was indeed observed for both TSC and NaHMP in Fig. 1, where at all pH levels studied, Ca-ion activity decreased progressively with increasing concentration of Ca-sequestrant up to an added level of approximately 60 mEq L^{-1}. In samples without added Ca-sequestrant, an effect of pH was noted, with lower Ca-ion activity at higher pH, but with increasing levels of sequestrant, such effects become progressively smaller, particularly for samples with added NaHMP (Fig. 1). Final levels of Ca-ion activity were lower for samples with added NaHMP than with added TSC (Fig. 1), which is in line with expectation based on association constants of Ca (Holt, Dalgleish, & Jenness, 1981) with both sequestrants. However, reductions in Ca-ion activity do not necessarily relate to micellar disruption. This is, e.g., the case for orthophosphates, which can complex with free Ca and reduce Ca-ion activity (De Kort et al., 2009), but where reductions in Ca-ion activity do not parallel micellar disruption (Culler, Saricay, & Harte, 2017; Mizuno & Lucey, 2005).

Micellar disruption though Ca-sequestrants can be monitored in various ways. Commonly used are measurement of turbidity or particle size. Reductions in turbidity indeed strongly correlated to casein micelle disruption (Pitkowski, Nicolai, & Durand, 2008), and, as shown in Fig. 2, both TSC and NaHMP led to strong reductions in turbidity at all pH values studied. Again, some initial differences in turbidity were observed as a function of pH, but these became less notable at higher levels of added sequestrant (Fig. 2). Reductions in turbidity were stronger for samples with added NaHMP than for

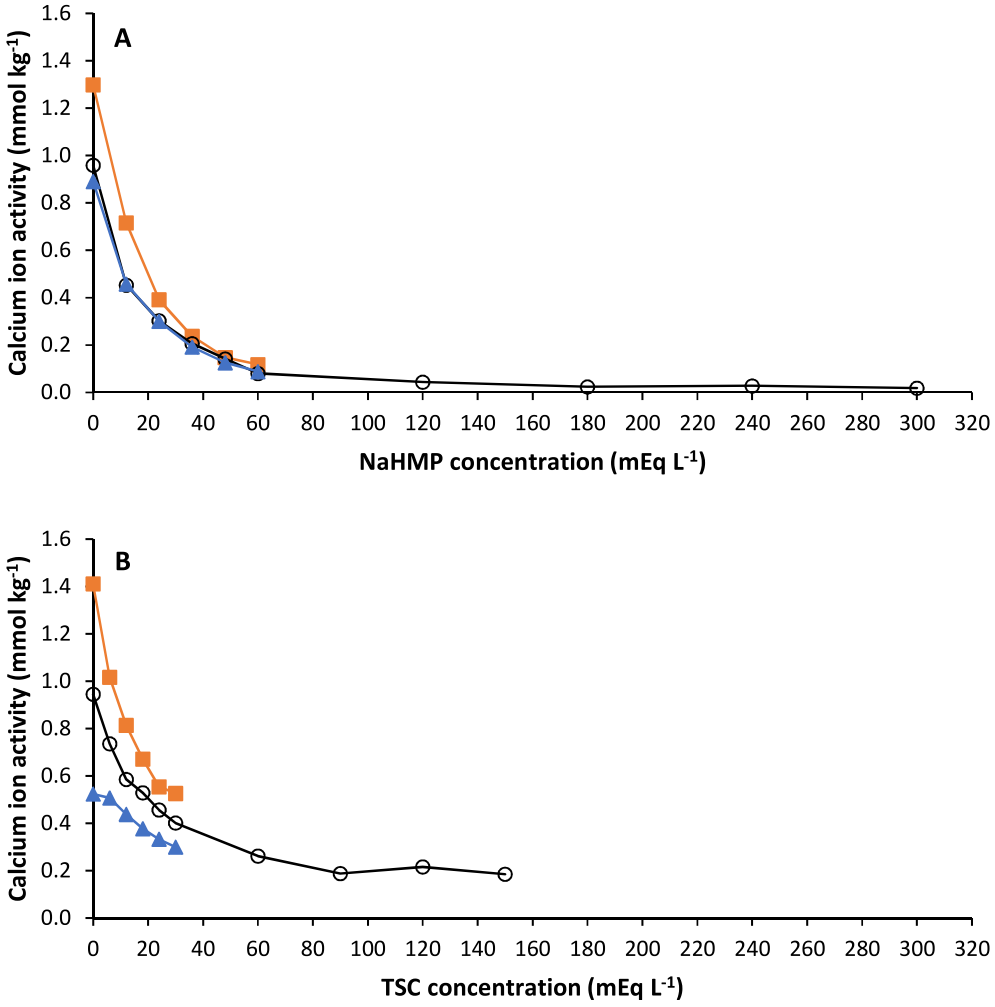

Fig. 1. Effect of sodium hexametaphosphate (NaHMP; A) or trisodium citrate (TSC; B) on the calcium ion activity of 9% MCI at pH 6.5 (■), 6.7 (○) or 6.9 (▲).

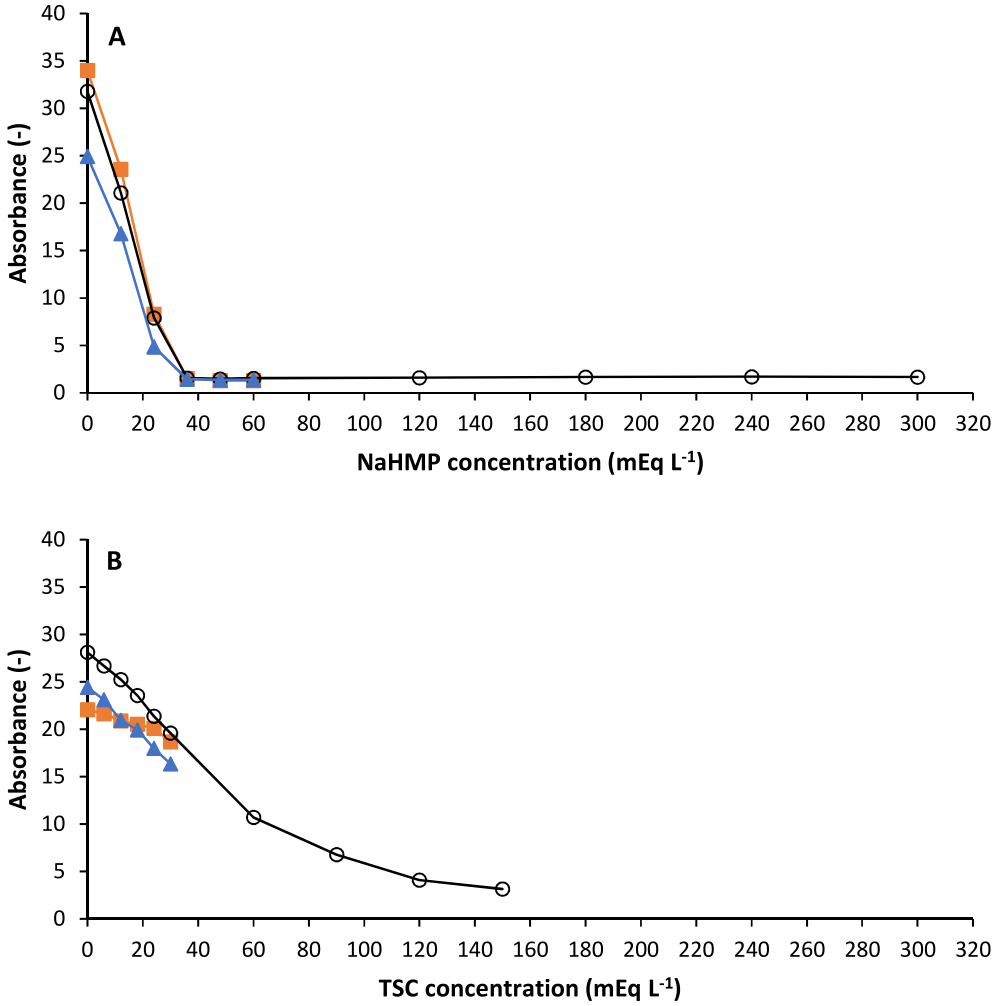

Fig. 2. Effect of sodium hexametaphosphate (NaHMP; A) or trisodium citrate (TSC; B) on the absorbance measured at 600 nm of 9% MCI at pH 6.5 (■), 6.7 (○) or 6.9 (▲).

samples with added TSC (Fig. 2), suggesting stronger micellar disruption for the former. Such changes in turbidity were paralleled by increases in levels of caseins non-sedimentable at 100,000 × *g* (Fig. 3), indicating that caseins were present in either free form or in clusters considerably smaller than casein micelles. Higher starting pH did result in higher levels of non-sedimentable caseins, which largely remained for samples with added TSC, but became less notable for samples with added NaHMP when addition level increased. Like for decreases in turbidity (Fig. 2), increases in non-sedimentable casein were more extensive for samples with added NaHMP than for samples with added TSC (Fig. 3). Correlation analysis between non-sedimentable levels of different casein fractions (Fig. 3) did not highlight specific caseins increasing more in non-sedimentable fraction (data not shown).

Turbidity of suspensions of casein micelles is determined by the size and number of light scattering particles, as well as their refractive index (Huppertz, Smiddy, & De Kruif, 2007). A disruption of casein micelles through the action of calcium sequestering salts would thus lead to a reduction in turbidity, as is indeed observed (Fig. 2). Particle size, however, is notably harder to relate to micellar disruption due to the fact that size distribution determined by light scattering is strongly dominated by the larger particles in the size distribution. As a result, a small number of residual casein micelles, or other particles such as, e.g., fat globules, which will always be present in skimmed milk or MCI, dominate the particle size distribution. This is also clear from results in Fig. 4, which show that while turbidity decreases progressively with increasing levels of TSC or NaHMP added (Fig. 2), for particle size, a decrease in size is initially observed, but at higher concentrations of NaHMP an increase is observed (Fig. 4). For TSC, particle size hardly changed as a result of addition (Fig. 4), despite the fact that notable reductions in turbidity (Fig. 2) and increases in non-sedimentable casein (Fig. 3) were observed.

Reduced turbidity (Fig. 2), which, as outlined above, is indicative of micellar disruption, was strongly correlated with increases in non-micellar casein (Fig. 3); for both TSC addition and NaHMP a near-linear relationship between turbidity and non-sedimentable casein (Fig. 5A) was observed. Likewise, a near-linear relationship between turbidity and non-sedimentable calcium (Fig. 5B), as well as between non-sedimentable casein and non-sedimentable calcium (Fig. 5C) was observed. Such correlations are logical considering that for casein to be sedimentable under the conditions used, it should be in particles of micellar dimensions (e.g., diameter 100–200 nm). Under conditions (close to those) found naturally in milk (pH close to neutral, moderate ionic strength and Ca-ion activity), casein particles large enough to sediment cannot be formed from casein alone, but require the presence of calcium phosphate nanoclusters, or at least significant calcium-induced aggregation. Likewise, the non-sedimentable casein fraction may still contain associated calcium interacting with proteins (Bijl, Huppertz, van

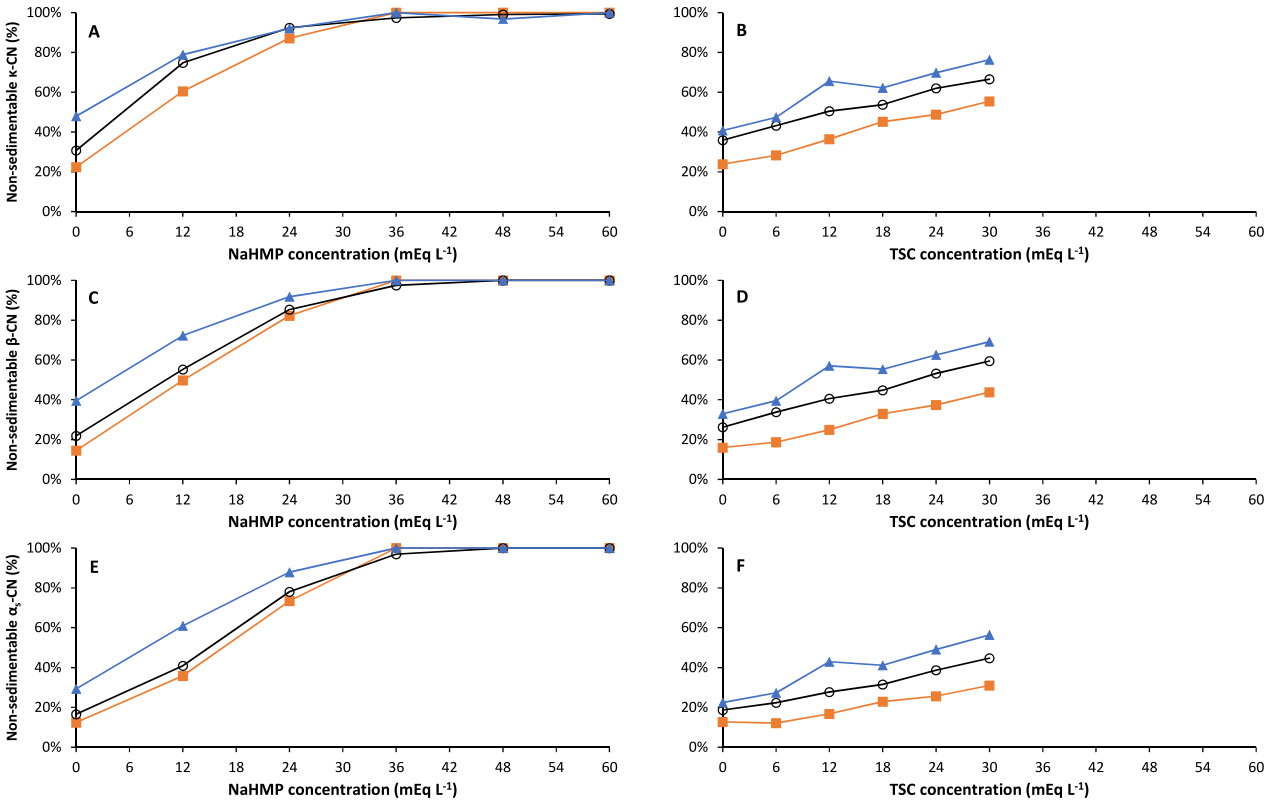

Fig. 3. Effect of sodium hexametaphosphate (NaHMP; A, C, E) or trisodium citrate (TSC; B, D, F) on the level of non-sedimentable κ-CN (A, B), β-CN (C, D) and α_s-CN (E, F) of 9% MCI at pH 6.5 (■), 6.7 (○) or 6.9 (▲).

Valenberg, & Holt, 2019). When dissociation of casein micelles is achieved via disruption of casein–casein interactions, this may even entail intact MCP nanoclusters, which appears to be the case e.g., for casein micelles disruption by urea (Aoki, Kako, & Imamura, 1986; Holt, 1998), deamidation (Miwa et al., 2010) and alkaline pH (Huppertz, Vaia, & Smiddy, 2008; Odagiri & Nickerson, 1965; Vaia et al., 2006).

Because the non-sedimentable fraction can include both soluble and protein-associated salts, it does not actually provide detailed information of the mechanism of disruption. Such information may be attained from (also) determining levels of calcium (and perhaps other salts) present in permeates or dialysates of treated samples where membranes were used for permeation of dissolved salts but prevent permeation of proteins, and the salts associated therewith. Vujicic et al. (1968) previously showed that when TSC (20 mmol L^{-1}) was added to milk, the level of 10 kDa permeable Ca increased strongly, from ~30% to ~65% of total Ca. In contrast, the addition of variety of phosphate salts, including orthophosphates, diphosphates and polyphosphates, did not increase levels of 10 kDa-permeable Ca, and in some cases even decreased it. Our experiments confirmed these findings (Table 2), showing that the addition of 12 or 24 mEq L^{-1} of NaHMP to MCI caused large increases in non-sedimentable Ca, but only small increases in 10 kDa-permeable Ca, whereas soluble Ca-HMP complexes should be small enough to permeate through a 10 kDa membrane. A further interesting note is seen when linking Ca distribution to casein distribution. At 24 mEq L^{-1} added NaHMP sedimentable Ca represents 19% of total Ca, whereas non-sedimentable non-10 kDa-permeable Ca represents 57% of total Ca (Table 2), so essentially an ~1:3 ratio. Now considering non-sedimentable caseins in this sample, ~80% of total casein is non-sedimentable, but ~20% was already soluble in the sample without added NaHMP (Fig. 4), so subtracting that also yields an ~1:3 ratio between casein that was sedimentable without added NaHMP and remained sedimentable and casein that has become non-sedimentable on adding 24 mEq L^{-1} of NaHMP. Hence, it appears that the Ca:casein ratio of the caseins that have become non-sedimentable is very similar to those that have remained sedimentable.

Therefore, from this work it appears while for the addition of TSC, micellar disruption arising from the sequestration of micellar Ca and the concomitant disappearance of MCP nanoclusters is a plausible mechanism, this is not the case for NaHMP-induced disruption of casein micelles, as Ca remains associated with the protein fraction. This suggests that the formation of soluble HMP-Ca complexes does not occur, or only to a limited extent. This is in line with data from Vujicic et al. (1968), who showed that the on addition of 25 mmol P in the form of NaHMP, 10 kDa-permeable P increased by only 5 mmol whereas 10 kDa non-permeable P increased by ~20 mmol L^{-1}. As a result, the Pi/Ca ratio in the 10 kDa non-permeable fraction increased strongly, from ~0.63 in control milk to ~1.42 in the milk with added NaHMP. This large increase in 10 kDa non-permeable Pi/Ca ratio is likely related to association of NaHMP with structural elements in the casein micelles.

Interactions of NaHMP with caseins has been suggested, and if present should occur with positively charged amino acid residues, e.g., Lys, Arg or His. Such association would lead to charge-reversal on residues, or sequences, where interactions occur, which could lead to dissociation. However, amino-HMP interactions are not likely to occur. Instead, it would be tempting to attribute the interaction of the NaHMP with the calcium phosphate nanoclusters. Two scenarios could be considered where the Ca remains 10 kDa-non-permeable, i.e., one through competition of HMP with

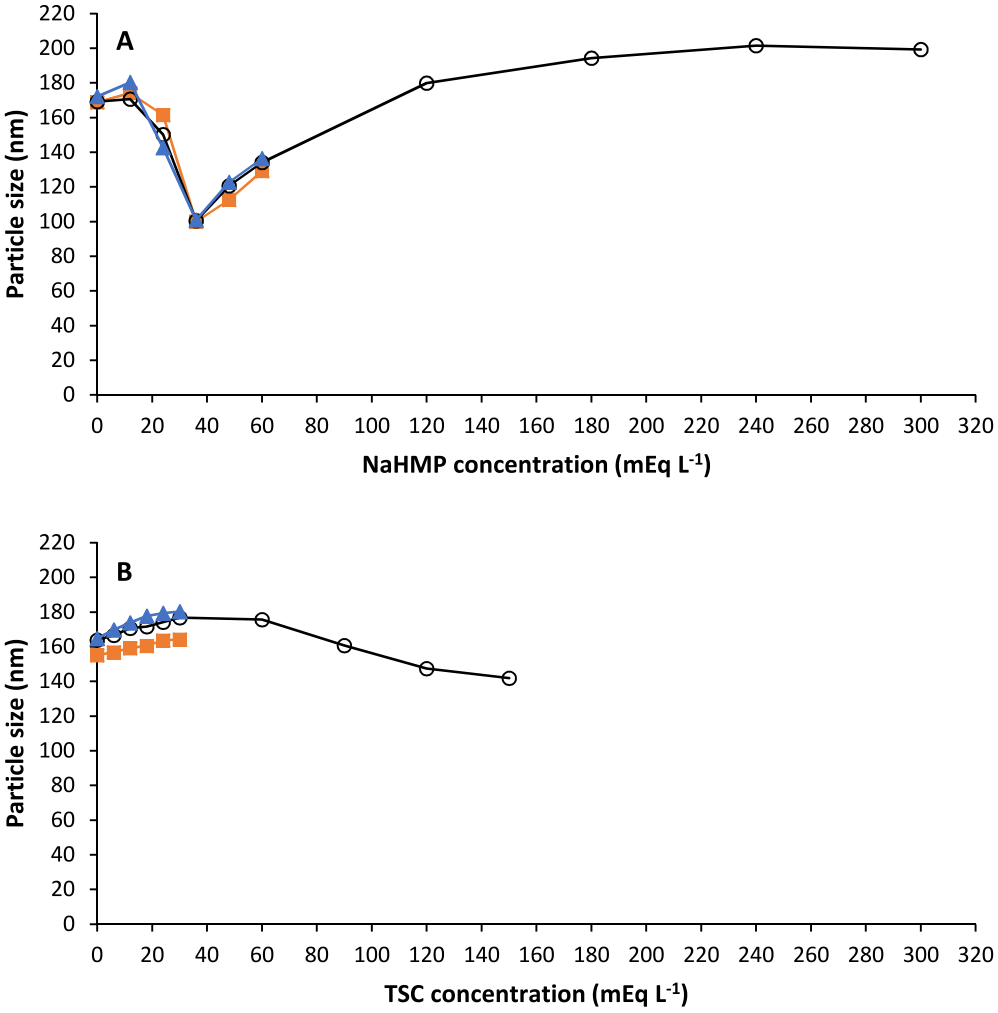

Fig. 4. Effect of sodium hexametaphosphate (NaHMP; A) or trisodium citrate (TSC; B) on the particle size of 9% MCI at pH 6.5 (■), 6.7 (○) or 6.9 (▲).

the SerP of the nanoclusters and one through competition of HMP with the Pi in the nanoclusters. In the first scenario, NaHMP could compete with the SerP-clusters on the surface of the MCP nanoclusters, thereby potentially displacing the SerP clusters and releasing nanoclusters from the micelles. This should not lead to increases in 10 kDa permeable Ca, as the nanoclusters are ~60–70 kDa, but should lead to an increase in non-sedimentable Ca, which is indeed observed (Table 2). In another scenario, HMP would compete with the P_i inside the nanoclusters, essentially leading to a peptisation process where, through displacement of some of the HMP with Pi, the nanoclusters essentially become fragmented in smaller entities, with HMP providing stabilisation of the newly formed surface. This, in turn, would lead to disruption of casein micelles.

Although further experimentation may be required to elucidate the mechanism of NaHMP-induced disruption of casein micelles, some indications can be taken from some of the data presented in this work. If HMP were to compete with SerP clusters, leading to a release of HMP-stabilised nanoclusters from the micelles, effects on the micelles would, in essence, not be dissimilar those observed for TSC. However, when considering effects of HMP on particle size (Fig. 4) and viscosity (Fig. 6), very different effects were observed. For NaHMP addition, viscosity (Fig. 6) increased very strongly, whereas for TSC addition, only a moderate increase in viscosity was observed. The former suggests the formation of highly swollen and/or elongated particles. This different behaviour on particle size and viscosity would make (partial) displacement of Pi rather than SerP in the nanocluster structure more likely. Consideration of association constants of Ca with either Pi or a SerP-SerP-SerP sequence would also make the displacement of Pi in the nanoclusters more likely as a mode of HMP-induced disruption of casein micelles.

Overall, from the findings of this work, it thus appears that modes of casein micelle dissociation via citrate and NaHMP may be very different. For TSC, solubilisation of MCP is the likely mode of action, whereas for HMP, peptisation is a more likely route, with the HMP acting as a peptisation agent. This does, however, draw attention to the importance of selecting the right techniques when studying modes of dissociation of casein micelles. Based on the conventionally applied measurements of turbidity, non-sedimentable caseins and non-sedimentable salts, one cannot distinguish between modes of action of TSC and NaHMP, nor does the inclusion of measurement of Ca-ion activity allow additional distinction. With the inclusion of also determining 10 kDa-permeable minerals, clear distinctions could be made.

This was also the case in studies on high pressure-induced dissociation of casein micelles by Regnault, Dumay, and Cheftel (2006). Prior studies had suggested HP-induced disruption were the result of HP-induced solubilisation of MCP based on increased levels of non-sedimentable calcium and phosphate (Huppertz, Fox, & Kelly, 2004). However, the studies by Regnault et al. (2006)

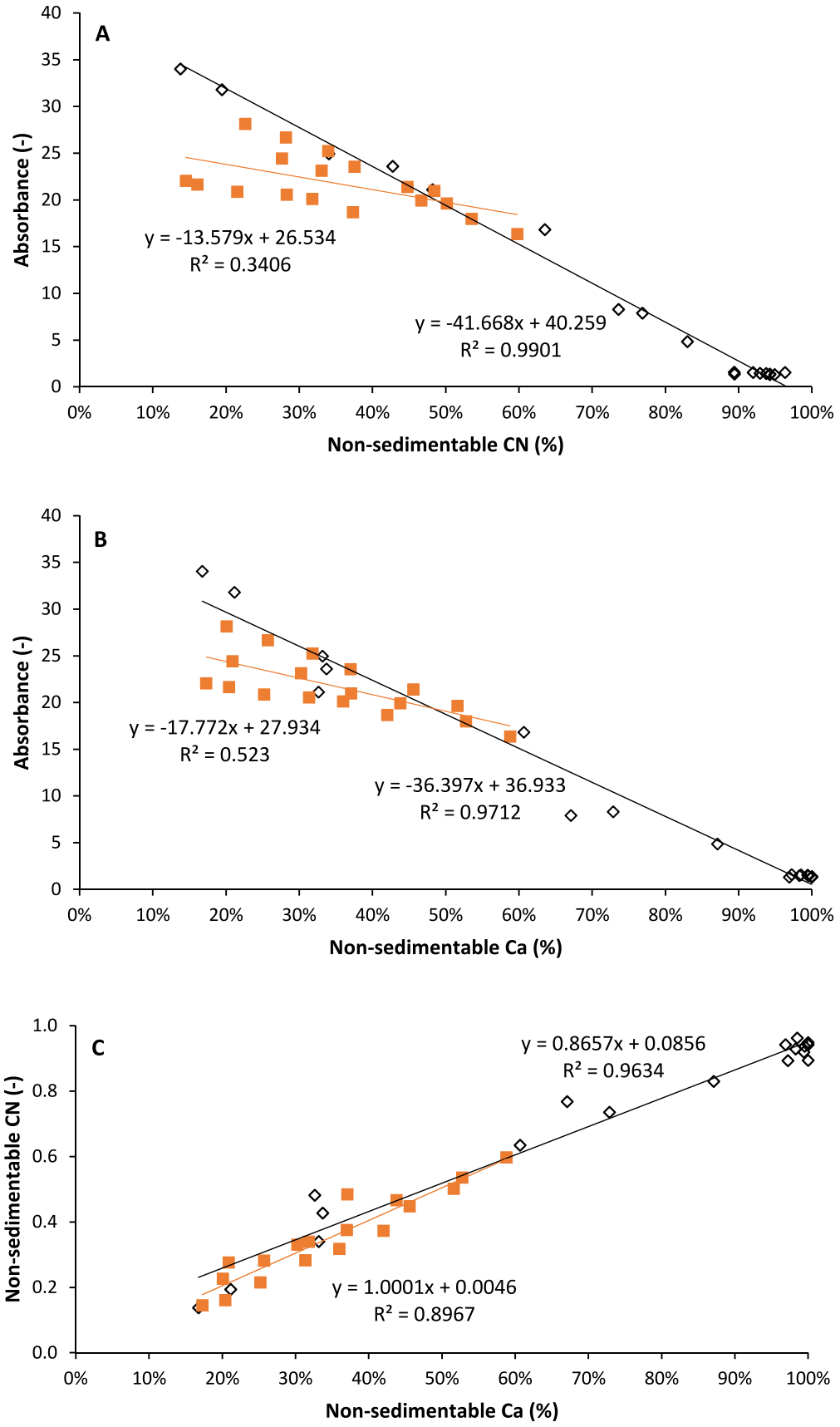

Fig. 5. Correlations between turbidity and non-sedimentable casein (A), turbidity and non-sedimentable calcium (B) and non-sedimentable casein and non-sedimentable Ca (C): sodium hexametaphosphate (◇); trisodium citrate (■).

Table 2
Calcium distribution in micellar casein isolate (MCI, 9% casein; pH 6.7) with 0, 12 or 24 mEq L^{-1} added sodium hexametaphosphate (NaHMP).[a]

NaHMP (mEq L^{-1})	Sedimentable Ca (%)	Non-sedimentable Ca (%)	10 kDa-permeable Ca (%)	Non-sedimentable 10 kDa non-permeable Ca (%)
0	77	23	11	12
12	53	47	17	30
24	19	81	24	57

[a] All values are expressed as a % of the total Ca in the sample.

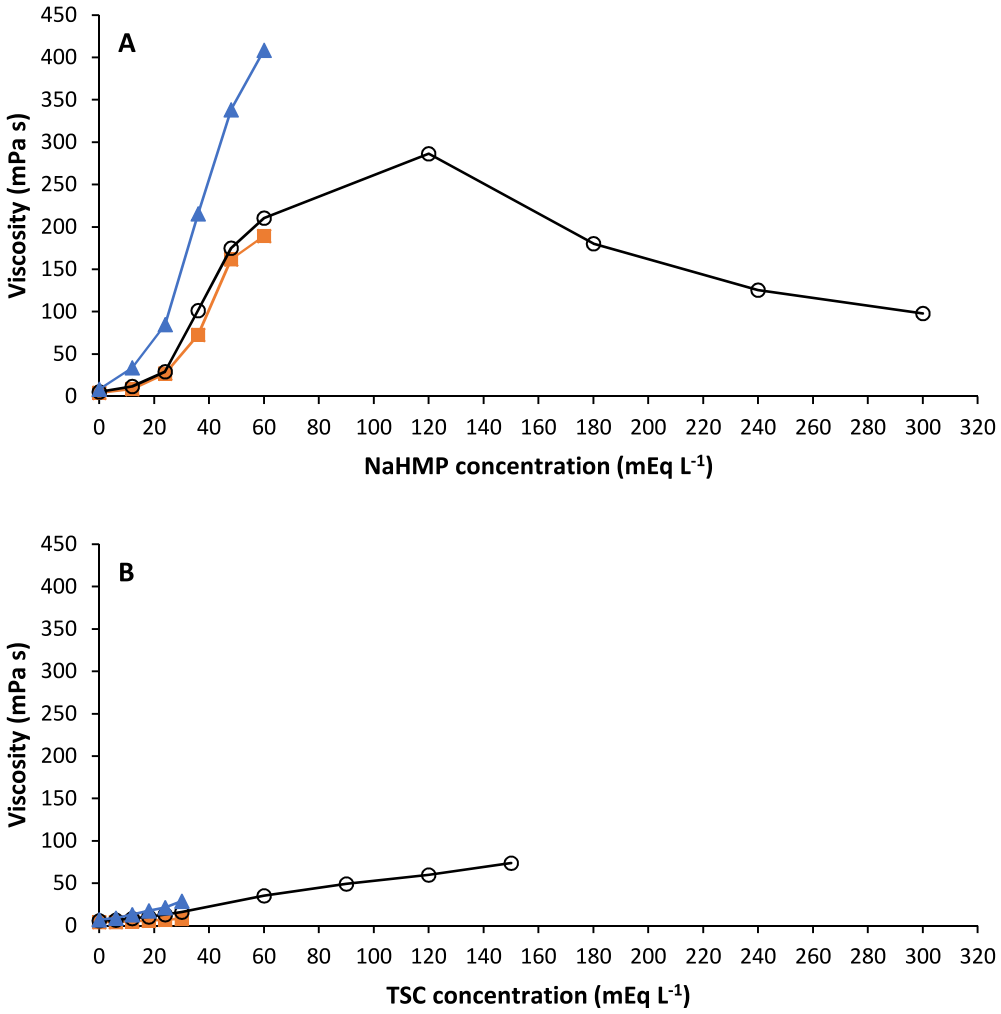

Fig. 6. Effect of sodium hexametaphosphate (NaHMP; A) or trisodium citrate (TSC; B) on the viscosity at 100 s^{-1} of 9% MCI at pH 6.5 (■), 6.7 (○) or 6.9 (▲).

showed that 10 kDa-permeable levels of Ca were not increased after HP treatment, in line with expectations based on the fact that mineral speciation equilibria in milk at atmospheric pressure cannot accommodate elevated levels of dissolved calcium and phosphate. Although it was later shown that solubilisation of MCP did occur at elevated pressure (Hubbard, Caswell, Lüdemann, & Arnold, 2002; Huppertz & De Kruif, 2007; Tromp, Huppertz, & Kohlbrecher, 2015) and was indeed the cause of disruption of micelles under pressure, such changes in mineral equilibria reversed on return to atmospheric pressure.

Considering other aforementioned causes of casein micelle dissociation, also those acting on casein interactions, i.e., deamidation (Miwa et al., 2010), pH increase (Huppertz et al., 2008; Odagiri & Nickerson, 1965; Vaia et al., 2006), addition of urea (Aoki et al., 1986; Holt, 1998; McGann & Fox, 1974; Smiddy et al., 2006) and addition of surfactants (Lefebvre-Cases et al., 1998) all decrease turbidity and increase non-sedimentable casein and calcium in milk. Hence, these parameters, which are closely linked essentially offer little more than the indication that micelles are indeed disrupted. For mechanistic insights, further analyses are required.

4. Conclusions

The results of this research confirm that NaHMP and TSC have the ability to disrupt the micelle structure but their effect on the physicochemical properties of micelles differs. Reduction in turbidity, changes in particle size, and increases in the levels of caseins and calcium in the non-sedimentable fraction clearly indicated that the micelle was (partially) disrupted. Presumably, TSC chelates calcium from MCP, however, for NaHMP, based on previous findings and in light of the findings obtained in this research, it can be suggested that the polyphosphate competes

with P_i from within the nanoclusters and partially displaces it, creating highly swollen and/or elongated particles and promoting the micelle disruption. The formation of these particles is likely to be responsible for the strong increase observed in the viscosity as well as the increase in the particle size observed over 36 mEq L^{-1}.

Declaration of competing interest

None.

Acknowledgement

The authors would like to thank the Public University of Navarra (Predoctoral grant Res. 1385/2017), Government of Navarra (Mobility grant n°: 0011-35642021-000015) and Erasmus+ for the financial support granted to Arantza Garcia. Open access funding provided by Public University of Navarra.

References

Anema, S. G. (2019). Age gelation, sedimentation, and creaming in UHT milk: A review. *Comprehensive Reviews in Food Science and Food Safety, 18*, 140–166.
Aoki, T., Kako, Y., & Imamura, T. (1986). Separation of casein aggregates cross-linked by colloidal calcium phosphate from bovine casein micelles by high performance gel chromatography in the presence of urea. *Journal of Dairy Research, 53*, 53–59.
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.
Broyard, C., & Gaucheron, F. (2015). Modifications of structures and functions of caseins: A scientific and technological challenge. *Dairy Science and Technology, 95*, 831–862.
Crowley, S. V., Megemont, M., Gazi, I., Kelly, A. L., Huppertz, T., & O'Mahony, J. A. (2014). Heat stability of reconstituted milk protein concentrate powders. *International Dairy Journal, 37*, 104–110.
Cruijsen, H., Poitevin, E., & Brunelle, S. L. (2019). Determination of minerals and trace elements in milk, milk products, infant formula, and adult nutrition: Collaborative study 2011.14 method modification. *Journal of AOAC International, 102*, 1845–1863.
Culler, M. D., Saricay, Y., & Harte, F. M. (2017). The effect of emulsifying salts on the turbidity of a diluted milk system with varying pH and protein concentration. *Journal of Dairy Science, 100*, 4241–4252.
Dalgleish, D. G., & Corredig, M. (2012). The structure of the casein micelle of milk and its changes during processing. *Annual Review of Food Science and Technology, 3*, 449–467.
Dalgleish, D. G., & Law, A. J. R. (1989). pH-Induced dissociation of bovine casein micelles. II. Mineral solubilization and its relation to casein release. *Journal of Dairy Research, 56*, 727–735.
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 and Technology, 89*, 283–299.
De Kort, E., Minor, M., Snoeren, T., Van Hooijdonk, T., & Van der Linden, E. (2012). Effect of calcium chelators on heat coagulation and heat-induced changes of concentrated micellar casein solutions: The role of calcium-ion activity and micellar integrity. *International Dairy Journal, 26*, 112–119.
Gaucheron, F. (2005). The minerals of milk. *Reproduction Nutrition Development, 45*, 473–483.
Holt, C. (1998). Casein micelle substructure and calcium phosphate interactions studied by sephacryl column chromatography. *Journal of Dairy Science, 81*, 2994–3003.
Holt, C., Dalgleish, D., & Jenness, R. (1981). Calculation of the ion equilibria in milk diffusate and comparison with experiment. *Analytical Biochemistry, 113*, 154–163.
Horne, D. S. (2008). Casein micelle structure and stability. In M. Boland, & H. Singh (Eds.), *Milk proteins* (pp. 133–162). San Diego, CA, USA: Academic Press.
Hubbard, C. D., Caswell, D., Lüdemann, H. D., & Arnold, M. (2002). Characterisation of pressure-treated skimmed milk powder dispersions: Application of NMR spectroscopy. *Journal of the Science of Food and Agriculture, 82*, 1107–1114.
Huppertz, T., & De Kruif, C. G. (2006). Disruption and reassociation of casein micelles under high pressure: Influence of milk serum composition and casein micelle concentration. *Journal of Agricultural and Food Chemistry, 54*, 5903–5909.
Huppertz, T., & De Kruif, C. G. (2007). High pressure-induced solubilisation of micellar calcium phosphate from cross-linked casein micelles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 295*, 264–268.
Huppertz, T., Fox, P. F., & Kelly, A. L. (2004). High pressure treatment of bovine milk: Effects on casein micelles and whey proteins. *Journal of Dairy Research, 71*, 97–106.
Huppertz, T., Fox, P. F., & Kelly, A. L. (2018). The caseins: Structure, stability, and functionality. In R. Y. Yada (Ed.), *Proteins in food processing* (2nd ed., pp. 49–92). Chichester, UK: Woodhead Publishing.
Huppertz, T., & Gazi, I. (2022). Caseins and casein micelles. In T. Huppertz, & T. Vasiljevic (Eds.), *Understanding and improving the functional and nutritional properties of milk* (pp. 155–185). Cambridge, UK: Burleigh Dodds Science Publishing Limited.
Huppertz, T., Gazi, I., Luyten, H., Nieuwenhuijse, H., Alting, A., & Schokker, E. (2017). Hydration of casein micelles and caseinates: Implications for casein micelle structure. *International Dairy Journal, 74*, 1–11.
Huppertz, T., Kelly, A. L., & De Kruif, C. G. (2006). Disruption and reassociation of casein micelles under high pressure. *Journal of Dairy Research, 73*, 294–298.
Huppertz, T., Smiddy, M. A., & De Kruif, C. G. (2007). Biocompatible micro-gel particles from cross-linked casein micelles. *Biomacromolecules, 8*, 1300–1305.
Huppertz, T., Vaia, B., & Smiddy, M. A. (2008). Reformation of casein particles from alkaline-disrupted casein micelles. *Journal of Dairy Research, 75*, 44–47.
Kapoor, R., & Metzger, L. E. (2008). Process cheese: Scientific and technological aspects—A review. *Comprehensive Reviews in Food Science and Food Safety, 7*, 194–214.
Le Graët, Y., & Gaucheron, F. (1999). pH-induced solubilization of minerals from casein micelles: Influence of casein concentration and ionic strength. *Journal of Dairy Research, 66*, 215–224.
Lefebvre-Cases, E., Gastaldi, E., & Tarodo De La Fuente, B. (1998). Influence of chemical agents on interactions in dairy products: Effect of SDS on casein micelles. *Colloids and Surfaces B: Biointerfaces, 11*, 281–285.
Lin, S. H. C., Leong, S. L., Dewan, R. K., Bloomfield, V. A., & Morr, C. V. (1972). Effect of calcium ion on the structure of native bovine casein micelles. *Biochemistry, 11*, 1818–1821.
McGann, T. C. A., & Fox, P. F. (1974). Physico-chemical properties of casein micelles reformed from urea-treated milk. *Journal of Dairy Research, 41*, 45–53.
Mekmene, O., Le Graët, Y., & Gaucheron, F. (2010). Theoretical model for calculating ionic equilibria in milk as a function of pH: Comparison to experiment. *Journal of Agricultural and Food Chemistry, 58*, 4440–4447.
Miwa, N., Yokoyama, K., Wakabayashi, H., & Nio, N. (2010). Effect of deamidation by protein-glutaminase on physicochemical and functional properties of skim milk. *International Dairy Journal, 20*, 393–399.
Mizuno, R., & Lucey, J. A. (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.
Odagiri, S., & Nickerson, T. A. (1965). Micellar changes in skimmilk treated with alkali or acid. *Journal of Dairy Science, 48*, 1157–1160.
Orlien, V., Knudsen, J. C., Colon, M., & Skibsted, L. H. (2006). Dynamics of casein micelles in skim milk during and after high pressure treatment. *Food Chemistry, 98*, 513–521.
Pitkowski, A., Nicolai, T., & Durand, D. (2008). Scattering and turbidity study of the dissociation of casein by calcium chelation. *Biomacromolecules, 9*, 369–375.
Power, O. M., Fenelon, M. A., O'Mahony, J. A., & McCarthy, N. A. (2019). Dephosphorylation of caseins in milk protein concentrate alters their interactions with sodium hexametaphosphate. *Food Chemistry, 271*, 136–141.
Regnault, S., Dumay, E., & Cheftel, J. C. (2006). Pressurisation of raw skim milk and of a dispersion of phosphocaseinate at 9 °C or 20 °C: Effects on the distribution of minerals and proteins between colloidal and soluble phases. *Journal of Dairy Research, 73*, 91–100.
Smiddy, M. A., Martin, J.-E. G. H., Kelly, A. L., De Kruif, C. G., & Huppertz, T. (2006). Stability of casein micelles cross-linked by transglutaminase. *Journal of Dairy Science, 89*, 1906–1914.
Tromp, R. H., Huppertz, T., & Kohlbrecher, J. (2015). Casein micelles at non-ambient pressure studied by neutron scattering. *Food Biophysics, 10*, 51–56.
Vaia, B., Smiddy, M. A., Kelly, A. L., & Huppertz, T. (2006). Solvent-mediated disruption of bovine casein micelles at alkaline pH. *Journal of Agricultural and Food Chemistry, 54*, 8288–8293.
Visser, S., Slangen, C. J., & Rollema, H. S. (1991). Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 548*, 361–370.
Vujicic, I., DeMan, J. M., & Woodrow, I. L. (1968). Interaction of polyphosphates and citrate with skim milk proteins. *Canadian Institute of Food Technology Journal, 1*, 17–21.
Walstra, P. (1990). On the stability of casein micelles. *Journal of Dairy Science, 73*, 1965–1979.
Xu, Y., Liu, D., Yang, H., Zhang, J., Liu, X., Regenstein, J. M., et al. (2016). Effect of calcium sequestration by ion-exchange treatment on the dissociation of casein micelles in model milk protein concentrates. *Food Hydrocolloids, 60*, 59–66.