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Influence of calcium-binding salts on heat stability and fouling of whey protein isolate dispersions

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ABSTRACT

The effect of the calcium-binding salts (CBS), trisodium citrate (TSC), tripotassium citrate (TPC) and disodium hydrogen phosphate (DSHP) at concentrations of 1–45 mM on the heat stability and fouling of whey protein isolate (WPI) dispersions (3%, w/v, protein) was investigated. The WPI dispersions were assessed for heat stability in an oil bath at 95 °C for 30 min, viscosity changes during simulated high-temperature short-time (HTST) and fouling behaviour using a lab-scale fouling rig. Adding CBS at levels of 5–30 mM for TSC and TPC and 25–35 mM for DSHP improved thermal stability of WPI dispersions by decreasing the ionic calcium (Ca^{2+}) concentration; however, lower or higher concentrations destabilised the systems on heating. Adding CBS improved heat transfer during thermal processing, and resulted in lower viscosity and fouling. This study demonstrates that adding CBS is an effective means of increasing WPI protein stability during HTST thermal processing.

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1. Introduction

Whey protein isolate (WPI) ingredients are widely used in food applications, including high-protein beverages, sports supplements and foods for special medical purposes (e.g., low-lactose and lactose-free products) due to their excellent nutritional and functional properties (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010). During manufacture of such products in which WPI ingredients are used, various heating regimes are typically applied such as pasteurisation, sterilisation and ultra-high temperature treatment to reduce the bacterial load and to inactivate enzymes that can impair quality during storage (Burton, 1988). Thus, WPI ingredients, and products made from them, commonly have to resist severe heat treatments. One of the consequences of poor heat stability of whey proteins is fouling on stainless steel surfaces during thermal processing. This can negatively impact production, affect product quality and increase production costs, as additional heating and pumping are needed to maintain the required temperature of the product and recover the drop in pressure during the process. Due to narrowing of the heat exchanger channels, the product has a shorter average residence time, which can impact product safety. Moreover, cleaning the deposit generated needs energy and substantial amount of water and chemicals (Goode, Asteriadou, Robbins, & Fryer, 2013).

Fouling is caused by unfavourable aggregation resulting from disulphide interchange reactions (-SH/SS), hydrophobic interactions and electrostatic shielding when whey protein-containing dispersions are heated to ≥75 °C (Sadeghinezhad et al., 2013). Of the whey proteins, β-lactoglobulin (β-lg) is the main protein responsible for fouling in milk and milk protein derivatives (Lalande & Rene, 1988). In addition, it has been established that ionic calcium (Ca^{2+}) can bind to whey proteins, promoting denaturation/aggregation and possible gelation on heating

(O'Kennedy & Mounsey, 2009; Petit, Herbig, Moreau, & Delaplace, 2011; Phan-Xuan et al., 2014; Ni et al., 2015; Joyce, Brodkorb, Kelly, & O'Mahony, 2017), thus causing deposit build-up during fouling (Jeurnink, Walstra, & de Kruif, 1996). Proteins generally deposit on heat transfer surfaces, in steel defects as well as inside the grain boundaries (Jimenez et al., 2013). In a fouling study using WPI dispersions (0.25–2.5%, w/w), Khaldi et al. (2018) reported that the calcium:protein molar ratio strongly influenced both denaturation of β-lg and fouling; the fouling deposit progressed from a thin and compacted structure at low calcium:protein ratio approaching a thicker and more open structure at higher calcium:protein ratio. In a study by Yang, Li, Chen & Mercadé-Prieto (2018), Ca^{2+} concentrations of 40–80 mg L^{-1} caused a 100-fold increase in the extent of fouling of WPI, even at the low temperatures tested (55 °C), as determined using a quartz crystal microbalance with dissipation monitoring (QCM-D) approach.

Ca^{2+}-induced heat instability, resulting in protein aggregation, is mainly caused by three factors/mechanisms (Simons, Kosters, Visschers, & de Jongh, 2002): (i) electrostatic shielding of negative charges on protein, thereby favouring hydrophobic bond-mediated aggregation; (ii) ion-specific hydrophobic interactions due to ion-induced conformational change; and (iii) crosslinking of adjacent anionic molecules by formation of protein- Ca^{2+}-protein bridges. To reduce the concentration of Ca^{2+}, calcium-binding salts (CBS) such as salts of phosphoric and citric acids can be added prior to processing to increase heat stability and reduce fouling (Lewis, 2011). The effects of CBS addition on protein structure and heat stability have been studied in various types of milk systems (Vujicic, de Man, & Woodrow, 1968), milk protein concentrate dispersions (McCarthy et al., 2017; Mizuno & Lucey, 2005), milk microfiltration concentrates (Toledo Renhe, Indris, & Corredig, 2018), concentrated micellar casein dispersions (De Kort, Minor, Snoeren, Van Hooijdonk, & Van der Linden, 2011, 2012), casein micelles (Nakajima,

Kawanishi, & Furuichi, 1975), individual caseins (Guo, Campbell, Chen, Lenhoff, & Velev, 2003), soymilk (Pathomrungsiyounggul, Lewis, & Grandison, 2010), reconstituted calcium-fortified milk powders (Williams, D'Ath, & Augustin, 2005) and whey protein beverages fortified with calcium (Keowmaneechai & McClements, 2002 & 2006).

Studies focusing specifically on improving heat stability of whey protein ingredients are scarce; in one such study, addition of sodium phosphate to a WPI solution containing 10% protein was shown to decrease protein-protein interactions and increase heat stability (Xiong, 1992). Another study using other CBS [ethylene glycol-bis, β-aminoethyl ether-N, N, N',N'-tetraacetic acid (EGTA) or ethylenediamine-tetraacetic acid (EDTA)] demonstrated the ability of these agents to reduce whey protein aggregation and gelation in 10% protein dispersions of whey protein concentrate (WPC) and WPI at pH 7 (Kuhn & Foegeding, 1991). Keowmaneechai and McClements (2006) incorporated EDTA and citrate (0–40 mM) in WPI-stabilised emulsions containing $CaCl_2$ to reduce or prevent whey protein aggregation; the authors reported that CBS sequestered Ca^{2+} when they were introduced at concentrations >3.5 mM EDTA or >5 mM citrate, thereby preventing droplet aggregation and improving emulsion stability by binding Ca^{2+}.

The objective of the present study was to determine the ability of different types and concentrations of CBS (trisodium citrate (TSC), tripotassium citrate (TPC) and disodium hydrogen phosphate (DSHP)) to increase the heat stability of WPI by binding Ca^{2+}, thereby reducing the propensity for fouling during HTST thermal processing. A more detailed understanding of the impact of CBS on the heat stability of whey protein dispersions is important as it will aid development of novel strategies to produce high heat stable whey protein ingredients for use in value added nutritional beverage applications.

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2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was obtained from Carbery Food Ingredients Ltd. (Ballineen, Co. Cork, Ireland). The WPI contained 94.0 g dry solids per 100 g powder, and in dry basis (w/w), 86.7% protein, 4.0% ash, 1.5% fat and 2.0% lactose. Trisodium citrate (TSC; $Na_3C_6H_5O_7.2H_2O$), tripotassium citrate (TPC; $C_6H_5K_3O_7.H_2O$) and disodium hydrogen phosphate (DSHP; $Na_2HPO_4.2H_2O$), hydrochloric acid (HCl), sodium hydroxide (NaOH), potassium chloride (KCl) and calcium chloride ($CaCl_2$) were of analytical grade and were sourced from Sigma-Aldrich (Arklow, Co. Wicklow, Ireland).

2.2. Preparation of whey protein dispersions

WPI powders were reconstituted to 3% (w/v) protein content in ultrapure water at 30 °C for 15 min using a Silverson high-speed laboratory shear mixer operating at 5000 rpm (Silverson model L4RT, Silverson, Chesham, UK). The dispersions were kept mixed using a laboratory overhead stirrer at low speed (300 rpm) for 2 h, to completely hydrate the powder particles and eliminate unwanted foam. The pH of the dispersions was measured before adding the CBS solutions. Concentrated solutions (1 M) of the CBS were prepared separately and added to the protein dispersions at this point to give final CBS concentrations of 1, 3, 5, 10, 15, 20, 25, 30, 35, 40 and 45 mM. The pH of the WPI dispersions with added CBS was adjusted to 6.4, the original pH of control WPI dispersions, and kept mixed under conditions described above for a further 2

h, after which pH was readjusted to 6.4, if required. The dispersions were then placed at 4 °C overnight with magnetic stirring to facilitate complete hydration and equilibration of minerals. After overnight hydration, the samples were equilibrated to 22 °C for 2 h, after which the pH was checked and re-adjusted to 6.4, if required.

2.3. Heat stability

The effect of CBS type and addition level, in the range 0–45 mM, on heat stability of the WPI dispersions was determined using the heat coagulation time (HCT) assay first described by Davies and White (1966), with some minor modifications. Samples (2.5 mL) were filled into glass tubes (length, 130 mm; external diameter, 10 mm; wall thickness, 2 mm) and placed in an oil bath (Hettich Elbanton Special Product, Hettich Benelux B.V., Geldermalsenat, the Netherlands) at 95 °C for 30 min, with gentle rocking at a rate of ~8 min^{-1}. Samples were retrieved, visually assessed for stability and were determined to be unstable if they displayed any flecks, flocs or particles of aggregated protein.

2.4. Simulated high-temperature short-time thermal treatment

Based on the results of analysis described in Section 2.3, selected WPI dispersions (28 g) with and without CBS, were heated (95 °C for 2 min) using an AR-G2 controlled stress rheometer (TA Instruments, Crawley, West Sussex, UK) equipped with a starch pasting cell (SPC) geometry, as reported by Drapala, Auty, Mulvihill, and O'Mahony (2016), to simulate a typical high-temperature short-time (HTST) thermal processing regime. WPI dispersions were

subjected to this simulated HTST treatment using the following CBS addition conditions: (1) no CBS, (2) WPI dispersions with 1 or 45 mM TSC, TPC and DSHP, and (3) the minimum concentration of each CBS (C_{min}) required to give stability on heating at 95 °C during 30 min (Section 2.3). Apparent viscosity (η) data at a shear rate of 15 s^{-1} was recorded at 1 s intervals during heating, holding and cooling, with this shear rate being chosen to prevent sample sedimentation or deposition on the geometry surface during heating (Drapala et al., 2016). Protein samples were recovered after heat treatment for analysis of pH, particle size, photographic imaging and ionic calcium concentration.

2.5. Particle size distribution

Particle size distribution (PSD) of the WPI dispersions (before and after heating) was measured using a laser light-diffraction unit (Mastersizer 3000, Malvern Instruments Ltd., Worcestershire, UK) equipped with a 300 RF (reverse Fourier) lens and He-Ne laser (λ of 633 nm), as detailed by Crowley et al. (2015). The PSD of samples with added CBS at 45 mM was not determined after heating, as these samples were gelled, and it was not possible to introduce such a highly structured sample to the Mastersizer. The samples were introduced to the dispersing unit using ultrapure water as a dispersant to reach an obscuration of 14% (± 1%). Analysis of PSD was performed using the non-spherical model, with particle refractive index of 1.46, absorption of 0.1 and dispersant refractive index of 1.33.

2.6. Measurement of pH and ionic calcium

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A standard pH meter (Meterlab®, Radiometer Analytical, Villeurbanne, Lyon, France), with PHM210 electrode, was used to measure pH at 20 °C. Ionic calcium (Ca^{2+}) of unheated and heated WPI dispersions (3%, w/v, protein at pH 6.4) was determined using a Ca^{2+}-ion selective electrode (Metrohm Ireland Ltd., Carlow, Ireland) as detailed by Crowley et al. (2014). Ca^{2+} was determined by means of a standard curve with Ca concentrations of 1, 2, 3, 4 or 5 mM.

2.7. Fouling behaviour of whey protein dispersions

Fouling behaviour of the WPI dispersions (1.5 L), using C_{min} of CBS required to give oil bath heat stability (5 mM TSC and TPC and 25 mM DSHP), was determined using a lab-scale fouling rig (Fig. 1). This fouling rig is a custom-built, stainless steel, tubular heat exchanger (THE) of length 586 mm and internal diameter 20 mm (Liam A. Barry Ltd., Cork, Ireland), connected to a circulating water-bath (Grant, LT ecocool™100, Cambridge, UK) which controlled temperature of the water used as the heating medium. Two electronic pressure transducers (PR-33X, Keller-druck, Dorchester, UK) located immediately before and after the THE were used to measure temperature and pressure in-line. The unit had a stainless steel feed vessel (capacity 3.0 L), connected directly to a positive displacement, progressive cavity pump (Torqueflow, Sydex, UK) and the liquid flow rate through the THE was controlled by means of a variable speed drive. The pressure on the recirculating WPI solution was set using a manual diaphragm throttling valve, with pressure recorded using an analogue pressure gauge (1107J486, WIKA, Los Angeles, CA, USA). Temperature of the protein dispersions was also measured using a thermocouple mounted to the feed vessel (Digitron 2024T Digital Thermometer Pt100, Port Talbot, UK).

The fouling system was operated at a laminar flow rate in batch recirculation mode. The protein feed solution was supplied at an initial temperature of 30 °C and the fouling experiments were conducted at 80 °C (temperature of the water heating medium) to simulate HTST thermal processing and preheating of spray dryer liquid concentrate feeds during ingredient manufacture and to enable sufficient denaturation and deposit build-up on the THE (Jimenez et al., 2013; Petit et al., 2011). The experiment was stopped when the change in temperature reached zero, which was after ~90 min of recirculation. During the experiment, temperature of the hot water and inlet and outlet protein dispersions from the THE were monitored by the pressure transducers located before and after the THE, and also using thermocouples. The initial back pressure was set at 1 bar using the throttling valve and was not adjusted during the runs. After the run, the protein dispersions were recovered and the system was cleaned in place (CIP) using a standardised CIP protocol, with detergents as follows. The system was first rinsed using deionised water (1.5 L), after which a caustic wash was performed using 1.5 L 1% NaOH solution (Ansep CIP, Ecolab, Co. Meath, Ireland) containing sodium hypochlorite (5%) at 64 °C over 20 min to dissolve fat, protein and carbohydrate deposits. After the caustic CIP step, the system was rinsed for 10 min with deionised water (1.5 L) to remove any residues of caustic. An acid wash was then performed using 1.5 L 1% acid solution (Horolith V, Ecolab, Co. Meath, Ireland) containing nitric acid (>30%) and orthophosphoric acid (<5%) at 46 °C for 30 min to dissolve any remaining carbohydrate and mineral deposits. After the acid wash the system was rinsed for 10 min with deionised water (1.5 L) to remove any residues of acid.

Individual samples of the flush water and CIP solutions were collected for analysis of protein and ash content. Total nitrogen was determined using the Kjeldahl method (AOAC official method no. 991.20; AOAC, 2005) and converted to protein using a conversion factor of

6.38. Ash content was determined by dry ashing in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany) at 500 °C for 5 h (AOAC official method no. 945.46; AOAC, 2005).

2.8. Statistical data analysis

All experiments were carried out using the same batch of whey protein isolate powder. Replicated measurements were carried out using freshly prepared WPI dispersions, with results presented as the means and standard deviations. One-way analysis of variance (one-way ANOVA) was carried out using Tukey's mean comparison test to find the significance in the differences between the mean values using the R Commander program V.2.15.0 (The R Foundation for Statistical Computing, Vienna, Austria) statistical analysis package, and the level of significance was determined at $p < 0.05$.

3. Results and discussion

3.1. Heat stability of whey protein dispersions with added calcium-binding salts

A stability mapping of WPI dispersions after heating in an oil bath at 95 °C for 30 min with the three CBS at various concentrations is given in Table 1; these initial screening test conditions were chosen to provide a harsh ‘stress test’ to differentiate between samples in terms of heat stability. This analysis of heat stability was performed to initially screen the effects of type and concentration of CBS on heat stability of WPI dispersions to facilitate selection of minimum concentration of each CBS required to give stability on heating at 95 °C for 30 min

(C_{min}) for each salt in refining the design of further analysis. Dispersions containing TSC and TPC exhibited wide regions of heat stability ranging from a minimum of 5 mM up to a maximum of 30 mM. In contrast, addition of DSHP exhibited a much narrower, and slightly higher, concentration stability region, as the C_{min} needed to achieve stability was 25 mM. The high concentration of DSHP required to stabilise the WPI system indicates that, using the studied conditions, citrate salts are more effective than phosphate salts in improving heat stability of WPI. Lower Ca^{2+} binding ability of DSHP, compared with citrate salts and other pyro- and poly-phosphates, has been observed for various dairy systems such as goat milk subjected to indirect UHT treatment (Chen, Grandison, & Lewis, 2012), rennet casein dispersions (McIntyre, O'Sullivan, & O'Riordan, 2016) and microfiltered milk concentrates (Toledo Renhe et al., 2018).

3.2. Viscosity during thermal processing

Following heat stability screening of WPI dispersions using the oil bath, three concentrations of each salt were used for further investigation using a high-temperature short-time (HTST) regime (95 °C for 2 min), to best simulate industrial HTST thermal processing and preheating of spray dryer liquid feeds during ingredient manufacture. Samples were heated in a starch pasting cell at a low shear rate of 15 s^{-1} to prevent sample sedimentation or deposition on the cell surface during heating (Drapala et al., 2016). WPI with extreme concentrations of each of the three CBS (1 or 45 mM, i.e., from the heat unstable region) and the minimum addition level of CBS required to stabilise the sample (C_{min}; i.e., 5 mM for TSC and TPC and 25 mM for DSHP) during screening were subjected to simulated HTST treatment at 95 °C for 2 min. A sample of WPI subjected to simulated HTST with no added CBS served as a control.

The apparent viscosity of WPI dispersions with and without added CBS during HTST treatment is presented in Fig. 2A,B. Initially, all samples were incubated at 15 °C prior to heating, with no significant differences ($p < 0.05$) in viscosity between samples (Table 2). During heating to 95 °C, a decrease in viscosity was recorded for most samples. This phenomenon is typical of milk protein systems containing whey protein, in which viscosity decreases on heating until a critical temperature (depending on protein content and profile), after which it increases due to heat induced changes in protein configuration (Joyce et al., 2017; Murphy, Fenelon, Roos, & Hogan, 2014). The initial decrease in viscosity of WPI solutions during heating may be attributed to decreases in the hydrodynamic size of whey protein molecules/particles resulting from increased strength of hydrophobic interactions (Horne, 1998). Such a decrease in viscosity prevails until a certain, protein-specific, temperature is reached, at which the protein structure changes irreversibly due to unfolding of proteins, disruption of hydrophobic interactions and other molecular interactions and aggregation, generally causing an increase in viscosity (Considine, Patel, Anema, Singh, & Creamer, 2007; Drapala et al., 2016).

During simulated HTST, heat stable samples (Fig. 2A) initially behaved similarly to unstable samples, undergoing a sudden, pronounced change in viscosity during holding at 95 °C; however, unlike the unstable samples, heat stable samples reverted towards pre-heating viscosity levels during subsequent holding and cooling. All stable samples were free of visible aggregates after heating (Fig. 2A) with no significant difference in viscosity before and after heating (Table 2). In contrast, for control and heat unstable samples, simulated HTST had a significant ($p < 0.05$) increase in final viscosity (Table 2), leading to protein aggregation (Fig. 2B).

Visual inspection after simulated HTST (Fig. 2A,B) showed large aggregates visible to the naked eye in the control sample, smaller aggregates at low CBS concentrations, clear

dispersions at the C_{min} of CBS and larger aggregates at the highest CBS concentrations. In addition to cross-linking, when Ca^{2+} ions bind to protein molecules, hydrophobic interactions are encouraged, which induces protein aggregation (Kohyama, Sano, & Doi, 1995). The large particles (Fig. 2B) obtained at the highest level of CBS addition should not be considered as primary whey protein aggregates, but as fragments of a gel network, which had been broken down while shearing in the starch pasting cell during heat treatment. In the pH range 6–7, and at a low ionic strength, the gel structure is formed by fine protein strands with thickness of only a few nanometers (Langton & Hermansson, 1992); however, the formation of these strands to form a complete gel is prohibited by the shear forces in the starch pasting cell and therefore, fine aggregates predominate.

Gelation of samples containing 45 mM CBS could be attributed to the ability of free ions, such as Na^{+} and HPO_4^{2-} in DSHP, to increase the ionic strength of the aqueous phase, resulting in reduced electrostatic repulsion between protein molecules. When the electrostatic repulsion is weakened (i.e., low surface charge density and/or high ionic strength), the increased hydrophobicity resulting from heat-induced unfolding of whey proteins on heating may be sufficiently strong for the attractive forces to dominate the repulsive forces, which promotes protein aggregation. This is in line with a previous study in a similar system (Keowmaneechai & McClements, 2002) using EDTA to improve thermal stability of WPI-stabilised oil-in-water emulsions containing calcium chloride. The authors reported that increasing the EDTA concentration up to 10 mM resulted in a stable protein system; however, protein aggregation occurred on further increasing the EDTA concentration, which was attributed to the increased ionic strength of the aqueous phase.

From these results, it can be concluded that the effect of CBS on heat stability is salt and concentration dependent. Xiong (1992) studied the effect of adding sodium dihydrogen phosphate (SDHP) on thermal aggregation of whey protein isolate heated at 1.6 °C min^{-1} from 25–96 °C. The author reported that low concentrations (5 mM) of SDHP at pH 6.0 greatly suppressed protein chain association and prevented aggregation; however, increasing the salt concentration to 20–50 mM promoted protein aggregation. Similar results have also been reported for whole milk systems; heat stability of goat milk subjected to ultra-high temperature (UHT) and in-container sterilisation treatments, with and without added CBS (TSC or DSHP) has been studied (Chen et al., 2012). As an example, adding DSHP at 6.4 mM decreased [Ca^{2+}] from 1.57 to 0.89 mM L^{-1}, with improved heat stability; however, increasing DSHP concentration to 12.8 mM further decreased [Ca^{2+}] to 0.59 mM L^{-1} while resulting in decreased heat stability. The authors reported that moderate reduction of [Ca^{2+}] concentration by adding 6.4 mM CBS prevented protein aggregation and sediment formation by increasing the negative charge on protein; however, protein aggregation and sediment formation was promoted when CBS concentration increased to 12.8 mM.

3.3. Particle size distribution

The particle size distributions (PSD) of unheated and heated WPI dispersions with and without added CBS are presented in Table 2 and Fig. 3. WPI dispersions with added citrate salts (TSC and TPC) at 5 mM showed very similar PSD profiles with no significant differences ($p < 0.05$), thus, only TSC at 5 mM was included in Fig. 3. Unheated WPI dispersions with no added CBS showed PSD (D_{50} and D_{90}) values (represents aggregate size in the 50 and 90% quantile of

337 the distribution, respectively) of 5.5 and 88 μm (Table 2). Similar PSD values (D_{50}) (16–40 μm)
338 have been reported by O'Loughlin, Murray, FitzGerald, Brodkorb, and Kelly (2014) using the
339 same source of WPI.

340 The measured particle size of the original WPI dispersions was relatively large, in
341 comparison with particle size results for WPI dispersions from previous studies (Drapala et al.,
342 2016; Mulcahy, Mulvihill, & O'Mahony, 2016); the latter authors reported an average volume
343 diameter (D4,3 value) of 1 μm in control WPI dispersions, which is relatively small compared
344 with a measured D4,3 for the control WPI sample in the present study of 17.7 μm. This relatively
345 large particle size may be attributed to the presence of a small proportion of high molecular
346 weight whey protein aggregates, as evidenced from protein profile analysis using sodium
347 dodecyl sulfate polyacrylamide gel electrophoresis under reducing and non-reducing conditions
348 (data not shown).

349 For the unheated WPI samples, a decrease in particle size was noted when CBS was
350 added. Adding low levels of TSC, TPC and DSHP (1 and 5 mM for TSC and TPC and 25 mM for
351 DSHP) reduced, in most cases, particle size of unheated samples (e.g., D_{90} = 6.1, 10.0 and 82.0
352 μm, respectively for the three CBSs' at 1 mM, compared with 88.0 μm for the unheated control).
353 Conversely, adding a high concentration (45 mM) of CBS (TPC and DSHP only) increased
354 significantly the mean particle size; this is in line with a previous study completed using a similar
355 system (Keowmaneechai & McClements, 2002), as discussed earlier (see Section 3.2).

356 Control samples showed a significant shift towards larger particle size after heating (Fig.
357 3A,G), as evidenced by a large peak in the 100–1000 μm size range, which was attributed to
358 aggregation. WPI dispersions with added CBS at the C_{min} (5 mM for TSC and TPC and 25 mM
359 for DSHP) showed a lesser extent of heat induced aggregation. The reduced aggregation on

heating in the CBS-containing samples was attributed to the binding of available Ca^{2+} by CBS, limiting protein cross-linking and increasing electrostatic repulsion between protein molecules (Pathomrungsiyounggul et al., 2010). Adding CBS (DSHP and TSC) has been shown to reduce the diameter of particles in soymilk (Pathomrungsiyounggul et al., 2010) and cows' milk (Tsioulpas, 2005) after heating. Toledo Renhe et al. (2018) reported that adding DSHP and TSC to fresh high protein microfiltered milk concentrates reduced particle size after heating at 120 °C over 10 min. PSD analysis also revealed differences in effectiveness of CBS; WPI dispersions containing 5 mM TSC demonstrated a small particle size with a monomodal distribution (Fig. 3 C, I), with a slight increase during simulated HTST (D_{90} = 5.00 and 6.90 µm for samples before and after heating, respectively), while the most stable DSHP sample (25 mM) demonstrated larger particle size values with a multimodal distribution (F and L) which displayed significant increases on heating (D_{90} = 152 and 265 µm before and after heating, respectively). Results of the present study showed that adding CBS at too high concentrations (45 mM) to WPI dispersions tended to destabilise (and gel) the system on heating at 95 °C for 2 min. Thus, PSD of samples with CBS added at 45 mM was not determined after heating, as these samples were gelled, and it was not possible to obtain a homogeneous sample for analysis.

3.4. pH and ionic calcium

The pH of WPI dispersions directly influences the charge on protein molecules/particles (and thereby electrostatic repulsion between same) and the concentration of free calcium ions in the serum phase (Walstra, Wouters, & Geurts, 2006). Before adding CBS, the pH of the WPI dispersions was ~6.4, while addition of the three CBS at different concentrations increased the

pH to values in the range 6.8 to 7.0, with the extent of pH change dependent on the type and concentration of CBS used (data not shown). Therefore, the pH of all samples was readjusted to 6.4 after addition of CBS to allow differentiation of the separate effects on heat stability of pH change and Ca^{2+} chelation.

Addition of CBS also decreased the [Ca^{2+}] of samples (Fig. 4B). The function of CBS is to reduce the chemical activity of metal ions, forming complexes with their unshared electron pair (Martell & Motekaitis, 2002), and [Ca^{2+}] decreased with increasing level of addition of CBS. In previous studies, increasing the addition levels of DSHP or TSC has also been shown to cause significant reduction in [Ca^{2+}] of raw cows' milk (Tsioulpas, 2005) and oil-in-water emulsions stabilised by whey protein isolate (Keowmaneechai & McClements, 2002). The capability of TSC and TPC to reduce [Ca^{2+}] was greater than that of DSHP; addition of TSC and TPC at 45 mM decreased the [Ca^{2+}] to very low levels ($\leq$ 0.02 mM L^{-1}).

The differences in the calcium-binding ability between citrates and phosphates in this study may be related to their high binding affinity at the pH of the study (6.4), which coincided with a basic pK value of TSC and TPC. In contrast, Mekmene and Gaucheron (2011) observed a higher calcium-binding affinity of pyrophosphate than citrate which was related to the pH of samples used in that study (8.1), which corresponded to a basic pK value for pyrophosphate. Adding CBS at concentrations up to 5 mM for TSC and TPC and 25 mM for DSHP led to progressive reductions in [Ca^{2+}] and restricted protein aggregation on heating (Fig. 4B). At addition levels greater that these respective values, while it was possible to reduce the [Ca^{2+}] further, aggregation of protein was considerably more pronounced, and heat stability decreased, with the reasons for this explained in detail in Section 3.2.

There was no significant difference in the pH of the control sample before and after heating (Fig. 4A), whereas, the pH of WPI samples containing CBS generally increased on heating. This behaviour was more pronounced in samples containing TSC and TPC than in those containing DSHP, and was unexpected for heated dairy systems where pH generally decreases on heating (Walstra et al., 2006). The effect of CBS on pH post heating was greatest at lower levels of addition of CBS. Toledo Renhe et al. (2018) reported similar results in concentrated micellar casein dispersions with different levels of citrates and phosphates. Both citrate salts at all levels of addition led to significantly higher pH post simulated HTST compared with the control while, adding DSHP resulted in higher pH post heating only at lower concentrations used (1 mM). In fact, the pH decreased on heating when higher concentrations (25 and 45 mM) of DSHP were used, which was more pronounced than the decrease in pH when citrate salts were added. Pathomrungsiyounggul et al. (2010) reported that there was a decrease in pH of soymilk with added DSHP after pasteurisation, while pH increased on pasteurisation of such milk with added TSC. This pronounced decrease in pH when DSHP was added corresponded to higher [Ca^{2+}], which may have contributed to the lower heat stability of these samples. Similar results have been reported by de Kort et al. (2012) for dispersions of micellar casein using sodium hexametaphosphate salt, in comparison with sodium citrate.

3.5. Fouling of whey protein dispersions

The temperature of the WPI dispersions on recirculation through the fouling rig with temperature at 80 °C with and without CBS added is shown in Fig. 5. Attempts to calculate the pressure drop were made; however, the temperature data were deemed more reliable, and are

428 therefore presented in this manuscript. The temperature of the control WPI solution increased
429 with recirculation through the fouling rig to reach 74.7 °C after 40 min. On progressive
430 recirculation, the temperature decreased to a final value of 73.3 °C by the end of the run. This
431 heat loss was likely due to build-up of deposits in the heat exchanger as can be seen from the
432 inset in Fig. 5. In contrast, WPI samples containing CBS maintained a constant temperature after
433 the initial heat-up time (i.e., between 40 and 90 min of running), indicating that addition of CBS
434 resulted in a considerably lower degree of fouling compared with the control. Furthermore, the
435 inset pictures in Fig. 5 show that after 90 min, the feed tank was heavily fouled in the control
436 sample, in comparison with samples with added CBS, in which it was clear.

437 The recovered protein dispersions and flush solutions obtained after applying the CIP
438 protocols were analysed (Table 3) for protein and ash content. This CIP regime was completed
439 once for each sample, except for the control sample where it had to be performed a second time
440 due to extensive fouling of the THE. Lower protein content was observed in the recovered
441 control sample, collected immediately after the run (1.81%, w/w), compared with samples
442 containing CBS (2.09, 2.24 and 2.12%, w/w) for TSC, TPC and DSHP, respectively,
443 demonstrating that protein was a significant contributor to fouling deposits (Fig. 5) in the control
444 sample. Significantly higher protein content was measured in the water flush for the control
445 sample compared with subsequent caustic and acid rinses, while very low ash content was
446 measured in the water flush for the control sample with significantly higher ($p < 0.05$) ash
447 contents measured in the caustic and acid rinses. The high fouling observed in the control sample
448 was due to the high [Ca^{2+}] (Fig. 4B), which facilitated whey protein aggregation and increased
449 particle size and viscosity during heating (Figs. 2 and 3). Simons et al. (2002) reported that
450 calcium could interact with the aspartic and glutamic acid carboxyl groups of β-lg, favouring the

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451 growth of fouling deposits. The significantly lower ash removal in the water rinse of the control
452 sample might be because the thick protein layer prevented removal of ash, which was
453 subsequently removed after caustic and acid rinses were applied.
454
455 **4. Conclusions**
456
457 This study demonstrated that the heat stability of whey protein isolate dispersions could
458 be increased using calcium-binding salts. Trisodium citrate and tripotassium citrate were more
459 effective in binding Ca^{2+}, and consequently increasing heat stability and reducing fouling, than
460 disodium hydrogen phosphate. Adding calcium-binding salts prevented fouling of the heat
461 exchanger, as evidenced from the improved heat transfer properties. This study showed that the
462 custom-fabricated fouling rig is a powerful tool to investigate the fouling and cleaning behaviour
463 of dairy heat exchangers. Its modular design facilitated the generation of deposits under HTST
464 conditions for compositional characterisation and evaluation of CIP protocol effectiveness. The
465 fouling rig could be used in the future to study different CIP detergent formulations and fouling
466 layer materials.
467
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Figure legends

Fig. 1. Schematic diagram of fouling rig with tubular heat exchanger (THE), feed vessel, positive displacement progressive cavity pump, two electronic pressure transducers located before and after the THE, circulating water bath for supplying the THE, thermocouples, flow throttling valve and an analogue pressure regulation valve to control pressure.

Fig. 2. Temperature (broken line) and viscosity (symbols) profiles of whey protein isolate (WPI) dispersions (3%, w/v, protein, pH 6.4) with and without calcium-binding salts (CBS) after applying a high temperature short-time (HTST) treatment at 95 °C for 2 min: (A) viscosity profiles of WPI dispersions without (—) and with CBS at concentrations conferring high heat stability, according to initial screening using the oil bath method; tripotassium citrate (TPC) at 5 mM (◆) and disodium hydrogen phosphate (DSHP) at 25 mM (▲) and (B) viscosity profiles of whey protein isolate dispersions without (—) and with CBS; tripotassium citrate (TPC) at 45 mM (◆) and disodium hydrogen phosphate (DSHP) at 1 mM (▲). Insets in the figures are photographs of WPI dispersions after heating.

Fig. 3. Particle size distribution before (A–F) and after (G–L) simulated high-temperature short-time (HTST) at 95 °C for 2 min of control whey protein isolate dispersions (3%, w/v, protein, pH 6.4) (A, G) and whey protein isolate dispersions with added trisodium citrate (TSC) at 1 mM (B, H) and 5 mM (C, I), tripotassium citrate (TPC) at 1 mM (D, J) and disodium hydrogen phosphate (DSHP) at 1 mM (E, K) and 25 mM (F, L).

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Fig. 4. pH (A) and ionic calcium concentration (B) of whey protein isolate dispersions (3%, w/v, protein, pH 6.4) before (■) and after (□) simulated high-temperature short-time treatment (HTST) at 95 °C for 2 min with and without calcium-binding salts.

Fig. 5. Temperature as a function of time for whey protein isolate dispersions (3%, w/v, protein, pH 6.4) without (■) and with calcium-binding salts (CBS): trisodium citrate, TSC (◆); tripotassium citrate, TPC (●); and disodium hydrogen phosphate, DSHP (▲) measured using electronic temperature measuring probes located immediately before and after the heat exchanger. Insets photographs show the feed tank for the control sample and sample with CBS after 1.5 h of running at 80 °C.

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Table 1

Mapping of heat stability of whey protein isolate dispersions. [a]

Salt	Calcium-binding salt concentration (mM)											
	0	1	3	5	10	15	20	25	30	35	40	45
Trisodium citrate	×	×	×	√	√	√	√	√	√	×	×	×
Tripotassium citrate	×	×	×	√	√	√	√	√	√	×	×	×
Disodium hydrogen phosphate	×	×	×	×	×	×	×	√	√	√	×	×

[a] Heat stability of whey protein isolate dispersions (3%, w/v, protein, pH 6.4) were measured using an oil bath at 95 °C for 30 min with the calcium-binding salts added at concentrations of 1–45 mM: √, stable solution after heating; ×, unstable solution after heating.

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Table 2

Viscosity before and after simulated high temperature short-time at 95 °C during 2 min and particle size distribution parameters of whey protein dispersions (3%, w/v, pH 6.4) with or without added calcium-binding salts (CBS). [a]

Sample	CBS concentration (mM)	Viscosity (mPa s)		D_{50} (µm)		D_{90} (µm)	
		Before heating	After heating	Before	After	Before	After
Control	0	12.8 ± 0.21^{a}	47.1 ± 3.20^{cd}*	5.50 ± 0.94^{a}	477 ± 83^{c}*	88.0 ± 15.6^{b}	744 ± 146^{c}*
TSC	1	12.6 ± 0.20^{a}	13.4 ± 0.01^{a}	3.50 ± 0.03^{a}	100 ± 17^{b}*	6.10 ± 0.17^{a}	193 ± 21^{ab}*
	5	12.7 ± 0.26^{a}	13.5 ± 0.16^{a}	3.10 ± 0.03^{a}	4.10 ± 0.6^{a}	5.00 ± 0.14^{a}	6.90 ± 1.7^{a}
	45	13.0 ± 0.20^{a}	59.0 ± 14.2^{d}*	2.60 ± 0.03^{a}	ND	3.62 ± 0.07^{a}	ND
TPC	1	12.8 ± 0.20^{a}	20.1 ± 5.32^{ab}*	4.10 ± 0.12^{a}	1241 ± 48^{e}*	10.0 ± 1.56^{a}	2412 ± 175^{d}*
	5	12.9 ± 0.19^{a}	13.8 ± 0.00^{a}	4.15 ± 0.23^{a}	3.94 ± 0.6^{a}	9.14 ± 1.18^{a}	9.34 ± 1.90^{a}
	45	13.0 ± 0.21^{a}	57.9 ± 8.85^{d}*	140 ± 6.25^{b}	ND	556 ± 5.47^{e}	ND
DSHP	1	12.8 ± 0.20^{a}	43.3 ± 12.0^{bd}*	5.10 ± 0.05^{a}	469 ± 15^{c}*	82.0 ± 3.60^{b}	843 ± 85^{c}*
	25	13.0 ± 0.21^{a}	15.9 ± 0.56^{a}	8.00 ± 0.67^{a}	93.0 ± 5.8^{b}*	152 ± 13.8^{c}	265 ± 13^{b}*
	45	12.9 ± 0.17^{a}	23.5 ± 0.24^{abc}*	146 ± 9.30^{b}	ND	262 ± 31.6^{d}	ND

[a] Abbreviations are: TSC, trisodium citrate; TPC, tripotassium citrate; DSHP, disodium hydrogen phosphate; ND, particle size not determined as the samples were gelled after heating. Particle size distribution parameters are: D_{50}, particle size in the 50% quantile of the distribution; D_{90}, particle size in the 90% quantile of the distribution. Values for a given WPI solution within a column not sharing a common superscript letter differ significantly ($p < 0.05$); values for a given WPI solution within a row with an asterisk signifies significant differences ($p < 0.05$) between the values measured before and after heating

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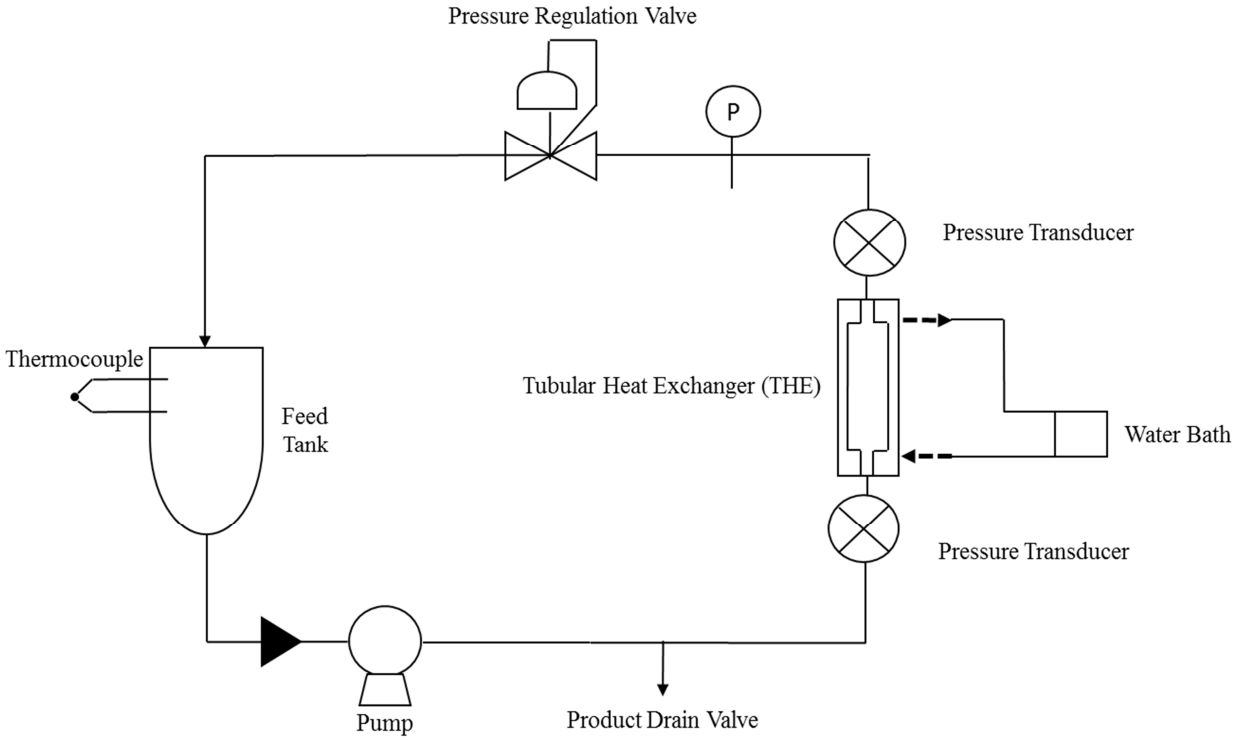
Table 3

Protein and ash content of dispersions recovered after applying a cleaning in place (CIP) protocol to the fouling rig. [a]

CBS type	CBS concentration (mM)	Analysis of CIP flush of fouling material (%, w/w)					
		Protein			Ash		
		Water	Caustic	Acid	Water	Caustic	Acid
Control	0	$0.65 \pm 0.18^{c,A}$	$0.25 \pm 0.07^{b,A}$	$0.07 \pm 0.01^{a,A}$	$0.00 \pm 0.00^{a,A}$	$0.12 \pm 0.03^{b,A}$	$0.26 \pm 0.06^{b,A}$
TSC	5	$0.40 \pm 0.01^{b,A}$	$0.40 \pm 0.03^{b,A}$	$0.04 \pm 0.01^{a,A}$	$0.04 \pm 0.03^{a,B}$	$0.18 \pm 0.02^{b,A}$	$0.16 \pm 0.04^{ab,A}$
TPC	5	$0.43 \pm 0.01^{c,A}$	$0.28 \pm 0.03^{b,A}$	$0.04 \pm 0.00^{a,A}$	$0.07 \pm 0.03^{a,B}$	$0.16 \pm 0.02^{a,A}$	$0.15 \pm 0.05^{a,A}$
DSHP	25	$0.54 \pm 0.06^{b,A}$	$0.46 \pm 0.10^{b,A}$	$0.05 \pm 0.01^{a,A}$	$0.12 \pm 0.00^{a,C}$	$0.18 \pm 0.00^{b,A}$	$0.21 \pm 0.01^{c,A}$

[a] Whey protein dispersions (3%, w/v, protein, pH 6.4) with or without added calcium-binding salts (CBS) were recovered after run and individual solutions were collected after applying water, caustic and acid rinses. Values for a given protein or ash value within a row not sharing a common lowercase superscript letter or within a column not sharing a common uppercase superscript letter differed significantly ($p < 0.05$).

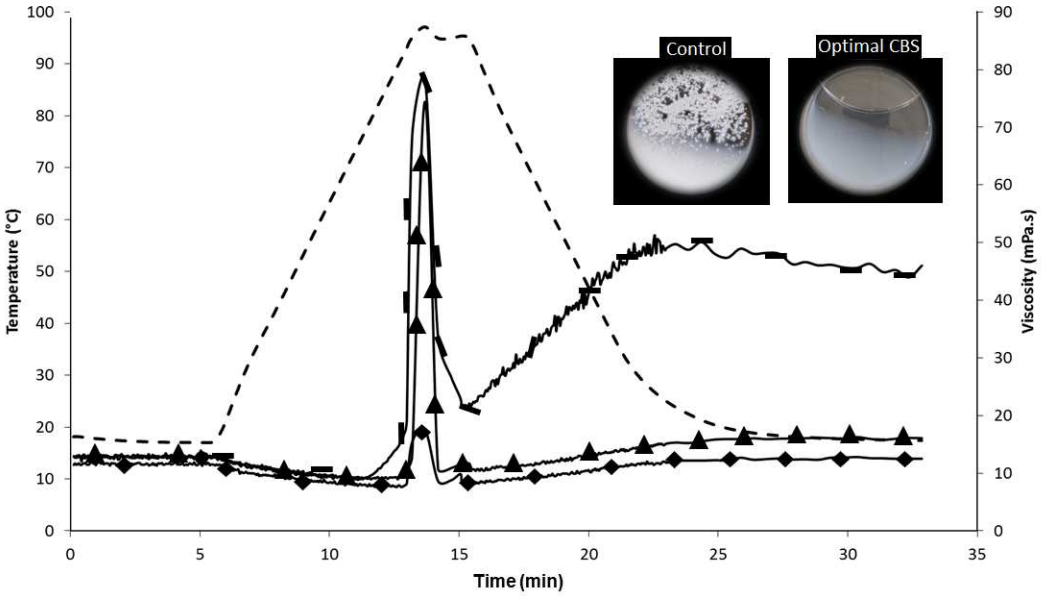
Fig. 1



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Fig. 2

(A)


Control
Optimal CBS
Temperature (°C)
Viscosity (mPa.s)
Time (min)

(B)

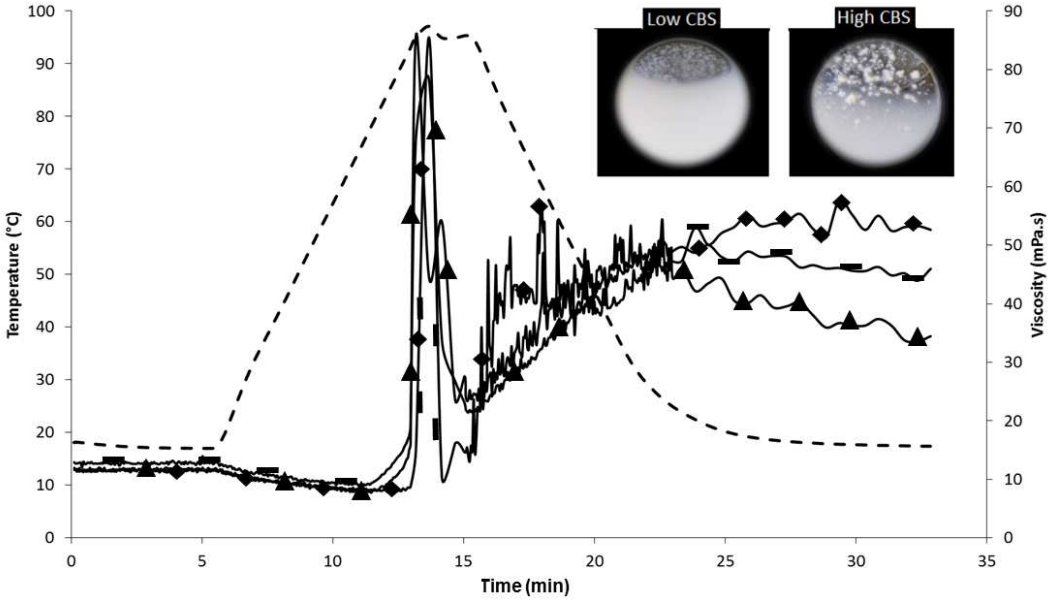

Low CBS
High CBS
Temperature (°C)
Viscosity (mPa.s)
Time (min)

Fig. 3

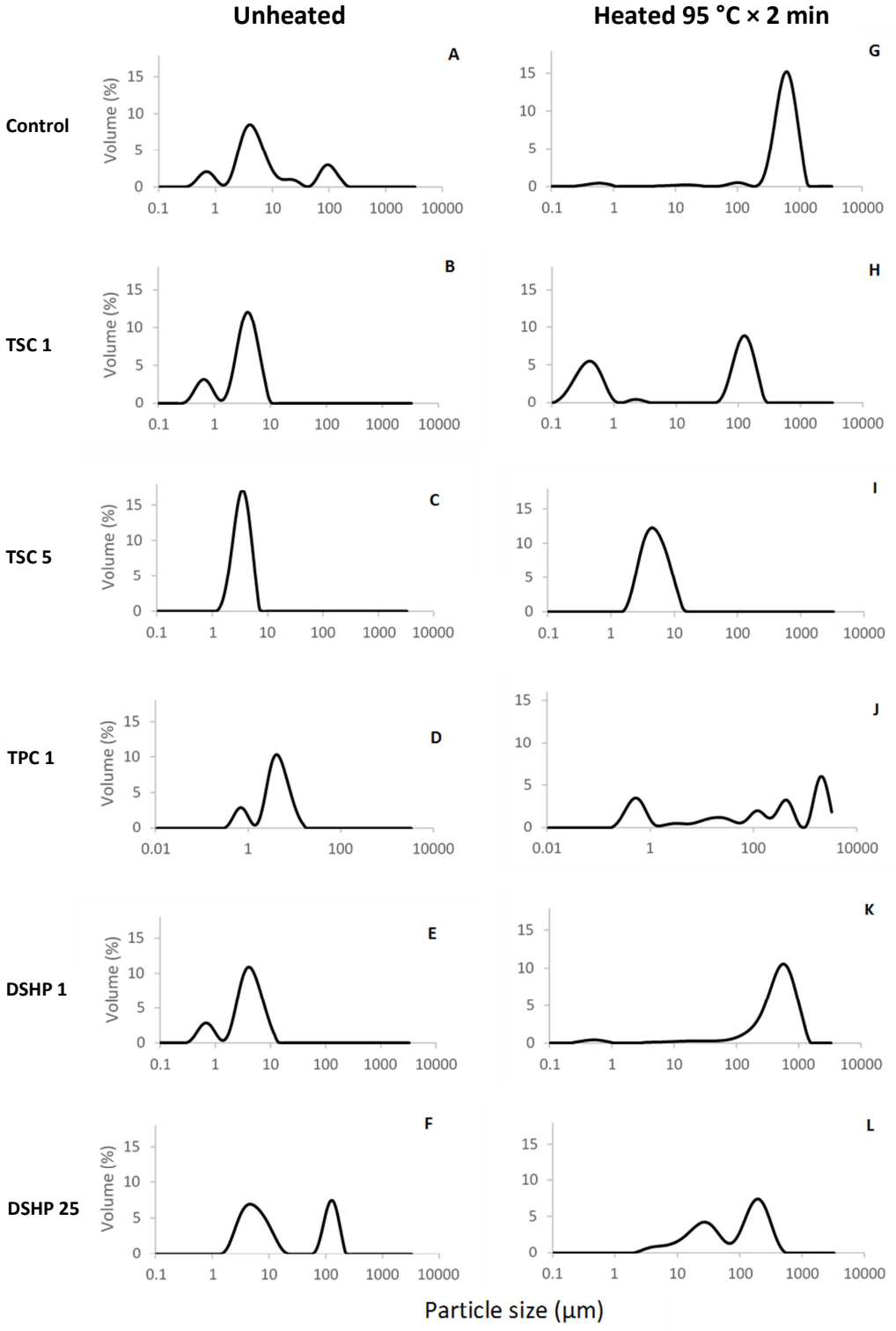

Unheated
Heated 95 °C × 2 min
Control
TSC 1
TSC 5
TPC 1
DSHP 1
DSHP 25
A
B
C
D
E
F
G
H
I
J
K
L
Volume (%)
Particle size (µm)

Fig. 4

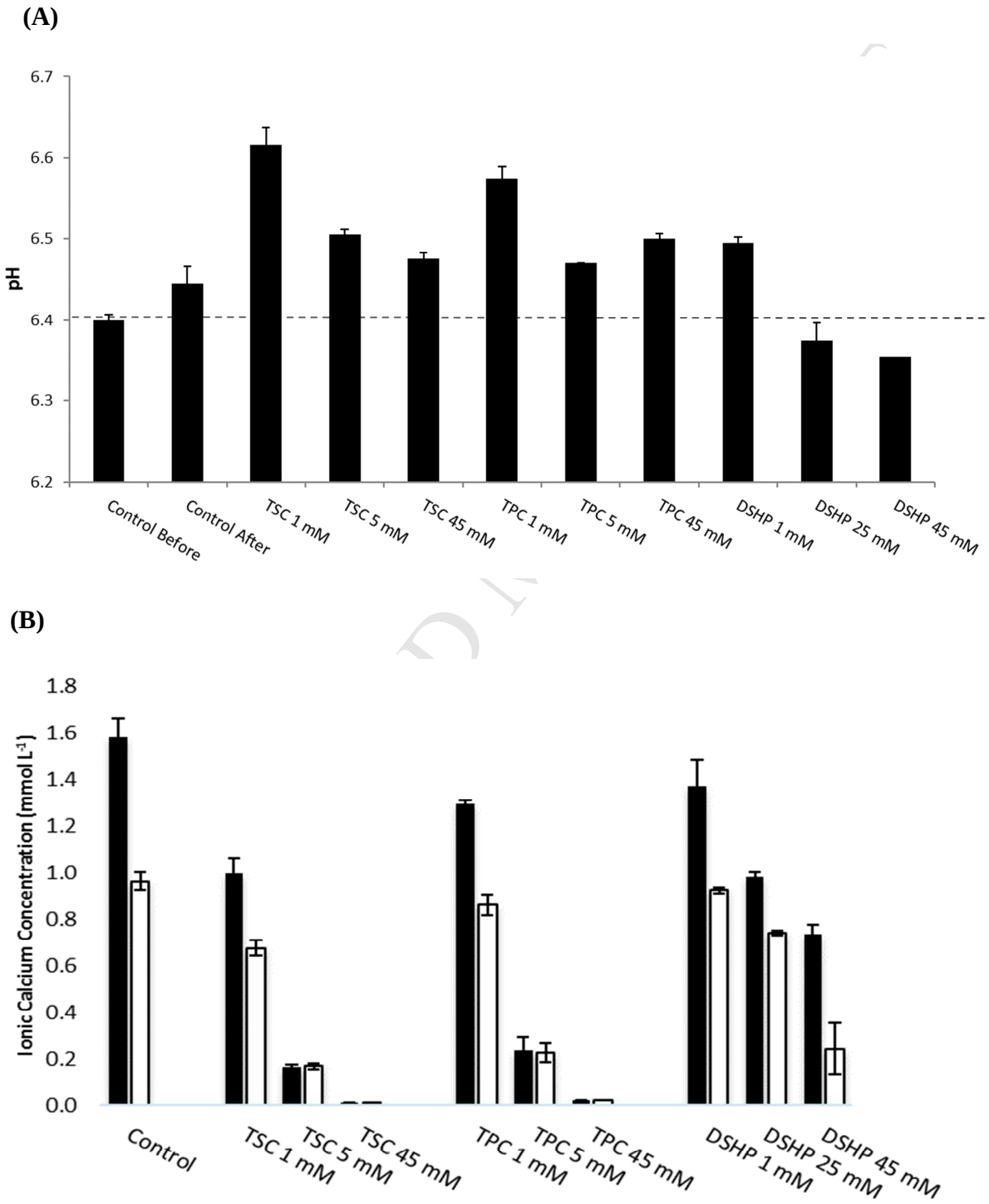
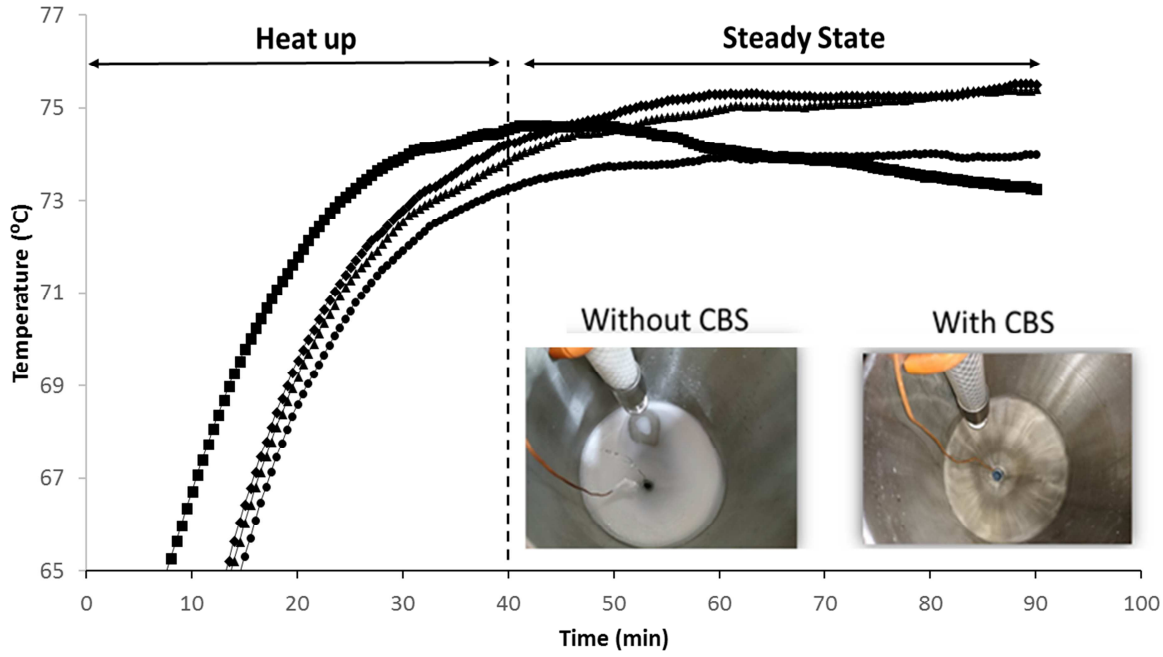

(A)
pH
6.7
6.6
6.5
6.4
6.3
6.2
Control Before
Control After
TSC 1 mM
TSC 5 mM
TSC 45 mM
TPC 1 mM
TPC 5 mM
TPC 45 mM
DSHP 1 mM
DSHP 25 mM
DSHP 45 mM
(B)
Ionic Calcium Concentration (mmol L-1)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
TSC 1 mM
TSC 5 mM
TSC 45 mM
TPC 1 mM
TPC 5 mM
TPC 45 mM
DSHP 1 mM
DSHP 25 mM
DSHP 45 mM

Fig. 5



ACCEPTED MANUSCRIPT