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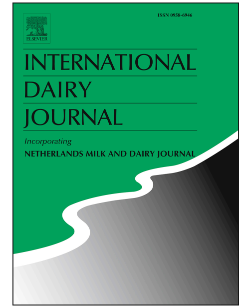

Influence of calcium-binding salts on heat stability and fouling of whey protein isolate dispersions

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2 **dispersions**
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24

25 ABSTRACT

26

27 The effect of the calcium-binding salts (CBS), trisodium citrate (TSC), tripotassium citrate
28 (TPC) and disodium hydrogen phosphate (DSHP) at concentrations of 1–45 mM on the heat
29 stability and fouling of whey protein isolate (WPI) dispersions (3%, w/v, protein) was
30 investigated. The WPI dispersions were assessed for heat stability in an oil bath at 95 °C for 30
31 min, viscosity changes during simulated high-temperature short-time (HTST) and fouling
32 behaviour using a lab-scale fouling rig. Adding CBS at levels of 5–30 mM for TSC and TPC and
33 25–35 mM for DSHP improved thermal stability of WPI dispersions by decreasing the ionic
34 calcium (Ca^{2+}) concentration; however, lower or higher concentrations destabilised the systems
35 on heating. Adding CBS improved heat transfer during thermal processing, and resulted in lower
36 viscosity and fouling. This study demonstrates that adding CBS is an effective means of
37 increasing WPI protein stability during HTST thermal processing.

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39 **1. Introduction**

40

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

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

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62 (O'Kennedy & Mounsey, 2009; Petit, Herbig, Moreau, & Delaplace, 2011; Phan-Xuan et al.,
63 2014; Ni et al., 2015; Joyce, Brodkorb, Kelly, & O'Mahony, 2017), thus causing deposit build-
64 up during fouling (Jeurnink, Walstra, & de Kruif, 1996). Proteins generally deposit on heat
65 transfer surfaces, in steel defects as well as inside the grain boundaries (Jimenez et al., 2013). In
66 a fouling study using WPI dispersions (0.25–2.5%, w/w), Khaldi et al. (2018) reported that the
67 calcium:protein molar ratio strongly influenced both denaturation of β-lg and fouling; the fouling
68 deposit progressed from a thin and compacted structure at low calcium:protein ratio approaching
69 a thicker and more open structure at higher calcium:protein ratio. In a study by Yang, Li, Chen &
70 Mercadé-Prieto (2018), Ca^{2+} concentrations of 40–80 mg L^{-1} caused a 100-fold increase in the
71 extent of fouling of WPI, even at the low temperatures tested (55 °C), as determined using a
72 quartz crystal microbalance with dissipation monitoring (QCM-D) approach.

73 Ca^{2+}-induced heat instability, resulting in protein aggregation, is mainly caused by three
74 factors/mechanisms (Simons, Kosters, Visschers, & de Jongh, 2002): (i) electrostatic shielding of
75 negative charges on protein, thereby favouring hydrophobic bond-mediated aggregation; (ii) ion-
76 specific hydrophobic interactions due to ion-induced conformational change; and (iii)
77 crosslinking of adjacent anionic molecules by formation of protein- Ca^{2+}-protein bridges. To
78 reduce the concentration of Ca^{2+}, calcium-binding salts (CBS) such as salts of phosphoric and
79 citric acids can be added prior to processing to increase heat stability and reduce fouling (Lewis,
80 2011). The effects of CBS addition on protein structure and heat stability have been studied in
81 various types of milk systems (Vujicic, de Man, & Woodrow, 1968), milk protein concentrate
82 dispersions (McCarthy et al., 2017; Mizuno & Lucey, 2005), milk microfiltration concentrates
83 (Toledo Renhe, Indris, & Corredig, 2018), concentrated micellar casein dispersions (De Kort,
84 Minor, Snoeren, Van Hooijdonk, & Van der Linden, 2011, 2012), casein micelles (Nakajima,

85 Kawanishi, & Furuichi, 1975), individual caseins (Guo, Campbell, Chen, Lenhoff, & Velev,
86 2003), soymilk (Pathomrungsiyounggul, Lewis, & Grandison, 2010), reconstituted calcium-
87 fortified milk powders (Williams, D'Ath, & Augustin, 2005) and whey protein beverages
88 fortified with calcium (Keowmaneechai & McClements, 2002 & 2006).

89 Studies focusing specifically on improving heat stability of whey protein ingredients are
90 scarce; in one such study, addition of sodium phosphate to a WPI solution containing 10%
91 protein was shown to decrease protein-protein interactions and increase heat stability (Xiong,
92 1992). Another study using other CBS [ethylene glycol-bis, β-aminoethyl ether-N, N, N',N'-
93 tetraacetic acid (EGTA) or ethylenediamine-tetraacetic acid (EDTA)] demonstrated the ability of
94 these agents to reduce whey protein aggregation and gelation in 10% protein dispersions of whey
95 protein concentrate (WPC) and WPI at pH 7 (Kuhn & Foegeding, 1991). Keowmaneechai and
96 McClements (2006) incorporated EDTA and citrate (0–40 mM) in WPI-stabilised emulsions
97 containing $CaCl_2$ to reduce or prevent whey protein aggregation; the authors reported that CBS
98 sequestered Ca^{2+} when they were introduced at concentrations >3.5 mM EDTA or >5 mM citrate,
99 thereby preventing droplet aggregation and improving emulsion stability by binding Ca^{2+}.

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

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108 **2. Materials and methods**

109

110 *2.1. Materials*

111

112 Whey protein isolate (WPI) was obtained from Carbery Food Ingredients Ltd. (Ballineen,
113 Co. Cork, Ireland). The WPI contained 94.0 g dry solids per 100 g powder, and in dry basis
114 (w/w), 86.7% protein, 4.0% ash, 1.5% fat and 2.0% lactose. Trisodium citrate (TSC;
115 $Na_3C_6H_5O_7.2H_2O$), tripotassium citrate (TPC; $C_6H_5K_3O_7.H_2O$) and disodium hydrogen
116 phosphate (DSHP; $Na_2HPO_4.2H_2O$), hydrochloric acid (HCl), sodium hydroxide (NaOH),
117 potassium chloride (KCl) and calcium chloride ($CaCl_2$) were of analytical grade and were
118 sourced from Sigma-Aldrich (Arklow, Co. Wicklow, Ireland).

119

120 *2.2. Preparation of whey protein dispersions*

121

122 WPI powders were reconstituted to 3% (w/v) protein content in ultrapure water at 30 °C
123 for 15 min using a Silverson high-speed laboratory shear mixer operating at 5000 rpm (Silverson
124 model L4RT, Silverson, Chesham, UK). The dispersions were kept mixed using a laboratory
125 overhead stirrer at low speed (300 rpm) for 2 h, to completely hydrate the powder particles and
126 eliminate unwanted foam. The pH of the dispersions was measured before adding the CBS
127 solutions. Concentrated solutions (1 M) of the CBS were prepared separately and added to the
128 protein dispersions at this point to give final CBS concentrations of 1, 3, 5, 10, 15, 20, 25, 30, 35,
129 40 and 45 mM. The pH of the WPI dispersions with added CBS was adjusted to 6.4, the original
130 pH of control WPI dispersions, and kept mixed under conditions described above for a further 2

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131 h, after which pH was readjusted to 6.4, if required. The dispersions were then placed at 4 °C
132 overnight with magnetic stirring to facilitate complete hydration and equilibration of minerals.
133 After overnight hydration, the samples were equilibrated to 22 °C for 2 h, after which the pH was
134 checked and re-adjusted to 6.4, if required.
135
136 *2.3. Heat stability*
137
138 The effect of CBS type and addition level, in the range 0–45 mM, on heat stability of the
139 WPI dispersions was determined using the heat coagulation time (HCT) assay first described by
140 Davies and White (1966), with some minor modifications. Samples (2.5 mL) were filled into
141 glass tubes (length, 130 mm; external diameter, 10 mm; wall thickness, 2 mm) and placed in an
142 oil bath (Hettich Elbanton Special Product, Hettich Benelux B.V., Geldermalsenat, the
143 Netherlands) at 95 °C for 30 min, with gentle rocking at a rate of ~8 min^{-1}. Samples were
144 retrieved, visually assessed for stability and were determined to be unstable if they displayed any
145 flecks, flocs or particles of aggregated protein.
146
147 *2.4. Simulated high-temperature short-time thermal treatment*
148
149 Based on the results of analysis described in Section 2.3, selected WPI dispersions (28 g)
150 with and without CBS, were heated (95 °C for 2 min) using an AR-G2 controlled stress
151 rheometer (TA Instruments, Crawley, West Sussex, UK) equipped with a starch pasting cell
152 (SPC) geometry, as reported by Drapala, Auty, Mulvihill, and O'Mahony (2016), to simulate a
153 typical high-temperature short-time (HTST) thermal processing regime. WPI dispersions were

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154 subjected to this simulated HTST treatment using the following CBS addition conditions: (1) no
155 CBS, (2) WPI dispersions with 1 or 45 mM TSC, TPC and DSHP, and (3) the minimum
156 concentration of each CBS (C_{min}) required to give stability on heating at 95 °C during 30 min
157 (Section 2.3). Apparent viscosity (η) data at a shear rate of 15 s^{-1} was recorded at 1 s intervals
158 during heating, holding and cooling, with this shear rate being chosen to prevent sample
159 sedimentation or deposition on the geometry surface during heating (Drapala et al., 2016).
160 Protein samples were recovered after heat treatment for analysis of pH, particle size,
161 photographic imaging and ionic calcium concentration.
162
163 *2.5. Particle size distribution*
164
165 Particle size distribution (PSD) of the WPI dispersions (before and after heating) was
166 measured using a laser light-diffraction unit (Mastersizer 3000, Malvern Instruments Ltd.,
167 Worcestershire, UK) equipped with a 300 RF (reverse Fourier) lens and He-Ne laser (λ of 633
168 nm), as detailed by Crowley et al. (2015). The PSD of samples with added CBS at 45 mM was
169 not determined after heating, as these samples were gelled, and it was not possible to introduce
170 such a highly structured sample to the Mastersizer. The samples were introduced to the
171 dispersing unit using ultrapure water as a dispersant to reach an obscuration of 14% (± 1%).
172 Analysis of PSD was performed using the non-spherical model, with particle refractive index of
173 1.46, absorption of 0.1 and dispersant refractive index of 1.33.
174
175 *2.6. Measurement of pH and ionic calcium*
176

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177 A standard pH meter (Meterlab®, Radiometer Analytical, Villeurbanne, Lyon, France),
178 with PHM210 electrode, was used to measure pH at 20 °C. Ionic calcium (Ca^{2+}) of unheated and
179 heated WPI dispersions (3%, w/v, protein at pH 6.4) was determined using a Ca^{2+}-ion selective
180 electrode (Metrohm Ireland Ltd., Carlow, Ireland) as detailed by Crowley et al. (2014). Ca^{2+} was
181 determined by means of a standard curve with Ca concentrations of 1, 2, 3, 4 or 5 mM.
182
183 *2.7. Fouling behaviour of whey protein dispersions*
184
185 Fouling behaviour of the WPI dispersions (1.5 L), using C_{min} of CBS required to give oil
186 bath heat stability (5 mM TSC and TPC and 25 mM DSHP), was determined using a lab-scale
187 fouling rig (Fig. 1). This fouling rig is a custom-built, stainless steel, tubular heat exchanger
188 (THE) of length 586 mm and internal diameter 20 mm (Liam A. Barry Ltd., Cork, Ireland),
189 connected to a circulating water-bath (Grant, LT ecocool™100, Cambridge, UK) which
190 controlled temperature of the water used as the heating medium. Two electronic pressure
191 transducers (PR-33X, Keller-druck, Dorchester, UK) located immediately before and after the
192 THE were used to measure temperature and pressure in-line. The unit had a stainless steel feed
193 vessel (capacity 3.0 L), connected directly to a positive displacement, progressive cavity pump
194 (Torqueflow, Sydex, UK) and the liquid flow rate through the THE was controlled by means of a
195 variable speed drive. The pressure on the recirculating WPI solution was set using a manual
196 diaphragm throttling valve, with pressure recorded using an analogue pressure gauge (1107J486,
197 WIKA, Los Angeles, CA, USA). Temperature of the protein dispersions was also measured
198 using a thermocouple mounted to the feed vessel (Digitron 2024T Digital Thermometer Pt100,
199 Port Talbot, UK).

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200 The fouling system was operated at a laminar flow rate in batch recirculation mode. The
201 protein feed solution was supplied at an initial temperature of 30 °C and the fouling experiments
202 were conducted at 80 °C (temperature of the water heating medium) to simulate HTST thermal
203 processing and preheating of spray dryer liquid concentrate feeds during ingredient manufacture
204 and to enable sufficient denaturation and deposit build-up on the THE (Jimenez et al., 2013; Petit
205 et al., 2011). The experiment was stopped when the change in temperature reached zero, which
206 was after ~90 min of recirculation. During the experiment, temperature of the hot water and inlet
207 and outlet protein dispersions from the THE were monitored by the pressure transducers located
208 before and after the THE, and also using thermocouples. The initial back pressure was set at 1
209 bar using the throttling valve and was not adjusted during the runs. After the run, the protein
210 dispersions were recovered and the system was cleaned in place (CIP) using a standardised CIP
211 protocol, with detergents as follows. The system was first rinsed using deionised water (1.5 L),
212 after which a caustic wash was performed using 1.5 L 1% NaOH solution (Ansep CIP, Ecolab,
213 Co. Meath, Ireland) containing sodium hypochlorite (5%) at 64 °C over 20 min to dissolve fat,
214 protein and carbohydrate deposits. After the caustic CIP step, the system was rinsed for 10 min
215 with deionised water (1.5 L) to remove any residues of caustic. An acid wash was then
216 performed using 1.5 L 1% acid solution (Horolith V, Ecolab, Co. Meath, Ireland) containing
217 nitric acid (>30%) and orthophosphoric acid (<5%) at 46 °C for 30 min to dissolve any
218 remaining carbohydrate and mineral deposits. After the acid wash the system was rinsed for 10
219 min with deionised water (1.5 L) to remove any residues of acid.
220 Individual samples of the flush water and CIP solutions were collected for analysis of
221 protein and ash content. Total nitrogen was determined using the Kjeldahl method (AOAC
222 official method no. 991.20; AOAC, 2005) and converted to protein using a conversion factor of

223 6.38. Ash content was determined by dry ashing in a muffle furnace (Nabertherm GmbH,
224 Lilienthal, Germany) at 500 °C for 5 h (AOAC official method no. 945.46; AOAC, 2005).
225
226 *2.8. Statistical data analysis*
227
228 All experiments were carried out using the same batch of whey protein isolate powder.
229 Replicated measurements were carried out using freshly prepared WPI dispersions, with results
230 presented as the means and standard deviations. One-way analysis of variance (one-way
231 ANOVA) was carried out using Tukey's mean comparison test to find the significance in the
232 differences between the mean values using the R Commander program V.2.15.0 (The R
233 Foundation for Statistical Computing, Vienna, Austria) statistical analysis package, and the level
234 of significance was determined at $p < 0.05$.
235
236 **3. Results and discussion**
237
238 *3.1. Heat stability of whey protein dispersions with added calcium-binding salts*
239
240 A stability mapping of WPI dispersions after heating in an oil bath at 95 °C for 30 min
241 with the three CBS at various concentrations is given in Table 1; these initial screening test
242 conditions were chosen to provide a harsh 'stress test' to differentiate between samples in terms
243 of heat stability. This analysis of heat stability was performed to initially screen the effects of
244 type and concentration of CBS on heat stability of WPI dispersions to facilitate selection of
245 minimum concentration of each CBS required to give stability on heating at 95 °C for 30 min

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246 (C_{min}) for each salt in refining the design of further analysis. Dispersions containing TSC and
247 TPC exhibited wide regions of heat stability ranging from a minimum of 5 mM up to a maximum
248 of 30 mM. In contrast, addition of DSHP exhibited a much narrower, and slightly higher,
249 concentration stability region, as the C_{min} needed to achieve stability was 25 mM. The high
250 concentration of DSHP required to stabilise the WPI system indicates that, using the studied
251 conditions, citrate salts are more effective than phosphate salts in improving heat stability of
252 WPI. Lower Ca^{2+} binding ability of DSHP, compared with citrate salts and other pyro- and poly-
253 phosphates, has been observed for various dairy systems such as goat milk subjected to indirect
254 UHT treatment (Chen, Grandison, & Lewis, 2012), rennet casein dispersions (McIntyre,
255 O'Sullivan, & O'Riordan, 2016) and microfiltered milk concentrates (Toledo Renhe et al., 2018).
256

257 *3.2. Viscosity during thermal processing*

258

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

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269 The apparent viscosity of WPI dispersions with and without added CBS during HTST
270 treatment is presented in Fig. 2A,B. Initially, all samples were incubated at 15 °C prior to
271 heating, with no significant differences ($p < 0.05$) in viscosity between samples (Table 2).
272 During heating to 95 °C, a decrease in viscosity was recorded for most samples. This
273 phenomenon is typical of milk protein systems containing whey protein, in which viscosity
274 decreases on heating until a critical temperature (depending on protein content and profile), after
275 which it increases due to heat induced changes in protein configuration (Joyce et al., 2017;
276 Murphy, Fenelon, Roos, & Hogan, 2014). The initial decrease in viscosity of WPI solutions
277 during heating may be attributed to decreases in the hydrodynamic size of whey protein
278 molecules/particles resulting from increased strength of hydrophobic interactions (Horne, 1998).
279 Such a decrease in viscosity prevails until a certain, protein-specific, temperature is reached, at
280 which the protein structure changes irreversibly due to unfolding of proteins, disruption of
281 hydrophobic interactions and other molecular interactions and aggregation, generally causing an
282 increase in viscosity (Considine, Patel, Anema, Singh, & Creamer, 2007; Drapala et al., 2016).
283 During simulated HTST, heat stable samples (Fig. 2A) initially behaved similarly to
284 unstable samples, undergoing a sudden, pronounced change in viscosity during holding at 95 °C;
285 however, unlike the unstable samples, heat stable samples reverted towards pre-heating viscosity
286 levels during subsequent holding and cooling. All stable samples were free of visible aggregates
287 after heating (Fig. 2A) with no significant difference in viscosity before and after heating (Table
288 2). In contrast, for control and heat unstable samples, simulated HTST had a significant ($p <$
289 0.05) increase in final viscosity (Table 2), leading to protein aggregation (Fig. 2B).
290 Visual inspection after simulated HTST (Fig. 2A,B) showed large aggregates visible to
291 the naked eye in the control sample, smaller aggregates at low CBS concentrations, clear

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

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

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314 From these results, it can be concluded that the effect of CBS on heat stability is salt and
315 concentration dependent. Xiong (1992) studied the effect of adding sodium dihydrogen
316 phosphate (SDHP) on thermal aggregation of whey protein isolate heated at 1.6 °C min^{-1} from
317 25–96 °C. The author reported that low concentrations (5 mM) of SDHP at pH 6.0 greatly
318 suppressed protein chain association and prevented aggregation; however, increasing the salt
319 concentration to 20–50 mM promoted protein aggregation. Similar results have also been
320 reported for whole milk systems; heat stability of goat milk subjected to ultra-high temperature
321 (UHT) and in-container sterilisation treatments, with and without added CBS (TSC or DSHP)
322 has been studied (Chen et al., 2012). As an example, adding DSHP at 6.4 mM decreased $[Ca^{2+}]$
323 from 1.57 to 0.89 mM L^{-1}, with improved heat stability; however, increasing DSHP concentration
324 to 12.8 mM further decreased $[Ca^{2+}]$ to 0.59 mM L^{-1} while resulting in decreased heat stability.
325 The authors reported that moderate reduction of $[Ca^{2+}]$ concentration by adding 6.4 mM CBS
326 prevented protein aggregation and sediment formation by increasing the negative charge on
327 protein; however, protein aggregation and sediment formation was promoted when CBS
328 concentration increased to 12.8 mM.

329

330 *3.3. Particle size distribution*

331

332 The particle size distributions (PSD) of unheated and heated WPI dispersions with and
333 without added CBS are presented in Table 2 and Fig. 3. WPI dispersions with added citrate salts
334 (TSC and TPC) at 5 mM showed very similar PSD profiles with no significant differences ($p <$
335 0.05), thus, only TSC at 5 mM was included in Fig. 3. Unheated WPI dispersions with no added
336 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

360 heating in the CBS-containing samples was attributed to the binding of available Ca^{2+} by CBS,
361 limiting protein cross-linking and increasing electrostatic repulsion between protein molecules
362 (Pathomrungsiyounggul et al., 2010). Adding CBS (DSHP and TSC) has been shown to reduce
363 the diameter of particles in soymilk (Pathomrungsiyounggul et al., 2010) and cows' milk
364 (Tsioulpas, 2005) after heating. Toledo Renhe et al. (2018) reported that adding DSHP and TSC
365 to fresh high protein microfiltered milk concentrates reduced particle size after heating at 120 °C
366 over 10 min. PSD analysis also revealed differences in effectiveness of CBS; WPI dispersions
367 containing 5 mM TSC demonstrated a small particle size with a monomodal distribution (Fig. 3
368 C, I), with a slight increase during simulated HTST (D_{90} = 5.00 and 6.90 µm for samples before
369 and after heating, respectively), while the most stable DSHP sample (25 mM) demonstrated
370 larger particle size values with a multimodal distribution (F and L) which displayed significant
371 increases on heating (D_{90} = 152 and 265 µm before and after heating, respectively). Results of
372 the present study showed that adding CBS at too high concentrations (45 mM) to WPI
373 dispersions tended to destabilise (and gel) the system on heating at 95 °C for 2 min. Thus, PSD
374 of samples with CBS added at 45 mM was not determined after heating, as these samples were
375 gelled, and it was not possible to obtain a homogeneous sample for analysis.
376
377 *3.4. pH and ionic calcium*
378
379 The pH of WPI dispersions directly influences the charge on protein molecules/particles
380 (and thereby electrostatic repulsion between same) and the concentration of free calcium ions in
381 the serum phase (Walstra, Wouters, & Geurts, 2006). Before adding CBS, the pH of the WPI
382 dispersions was ~6.4, while addition of the three CBS at different concentrations increased the

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383 pH to values in the range 6.8 to 7.0, with the extent of pH change dependent on the type and
384 concentration of CBS used (data not shown). Therefore, the pH of all samples was readjusted to
385 6.4 after addition of CBS to allow differentiation of the separate effects on heat stability of pH
386 change and Ca^{2+} chelation.

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

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

405 There was no significant difference in the pH of the control sample before and after
406 heating (Fig. 4A), whereas, the pH of WPI samples containing CBS generally increased on
407 heating. This behaviour was more pronounced in samples containing TSC and TPC than in those
408 containing DSHP, and was unexpected for heated dairy systems where pH generally decreases
409 on heating (Walstra et al., 2006). The effect of CBS on pH post heating was greatest at lower
410 levels of addition of CBS. Toledo Renhe et al. (2018) reported similar results in concentrated
411 micellar casein dispersions with different levels of citrates and phosphates. Both citrate salts at
412 all levels of addition led to significantly higher pH post simulated HTST compared with the
413 control while, adding DSHP resulted in higher pH post heating only at lower concentrations used
414 (1 mM). In fact, the pH decreased on heating when higher concentrations (25 and 45 mM) of
415 DSHP were used, which was more pronounced than the decrease in pH when citrate salts were
416 added. Pathomrungsiyounggul et al. (2010) reported that there was a decrease in pH of soymilk
417 with added DSHP after pasteurisation, while pH increased on pasteurisation of such milk with
418 added TSC. This pronounced decrease in pH when DSHP was added corresponded to higher
419 [Ca^{2+}], which may have contributed to the lower heat stability of these samples. Similar results
420 have been reported by de Kort et al. (2012) for dispersions of micellar casein using sodium
421 hexametaphosphate salt, in comparison with sodium citrate.
422
423 *3.5. Fouling of whey protein dispersions*
424
425 The temperature of the WPI dispersions on recirculation through the fouling rig with
426 temperature at 80 °C with and without CBS added is shown in Fig. 5. Attempts to calculate the
427 pressure drop were made; however, the temperature data were deemed more reliable, and are

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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

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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469

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477

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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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1 **Table 1**

2 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	×	×	×	×	×	×	×	√	√	√	×	×

3

4 [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

5 with the calcium-binding salts added at concentrations of 1–45 mM: √, stable solution after heating; ×, unstable solution after heating.

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6 **Table 2**

7 Viscosity before and after simulated high temperature short-time at 95 °C during 2 min and particle size distribution parameters of
8 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

9

10

11 [a] Abbreviations are: TSC, trisodium citrate; TPC, tripotassium citrate; DSHP, disodium hydrogen phosphate; ND, particle size not
12 determined as the samples were gelled after heating. Particle size distribution parameters are: D_{50}, particle size in the 50% quantile of
13 the distribution; D_{90}, particle size in the 90% quantile of the distribution. Values for a given WPI solution within a column not sharing
14 a common superscript letter differ significantly ($p < 0.05$); values for a given WPI solution within a row with an asterisk signifies
15 significant differences ($p < 0.05$) between the values measured before and after heating
16

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17

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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 ($p < 0.05$).

ACCEPTED MANUSCRIPT

Fig. 1

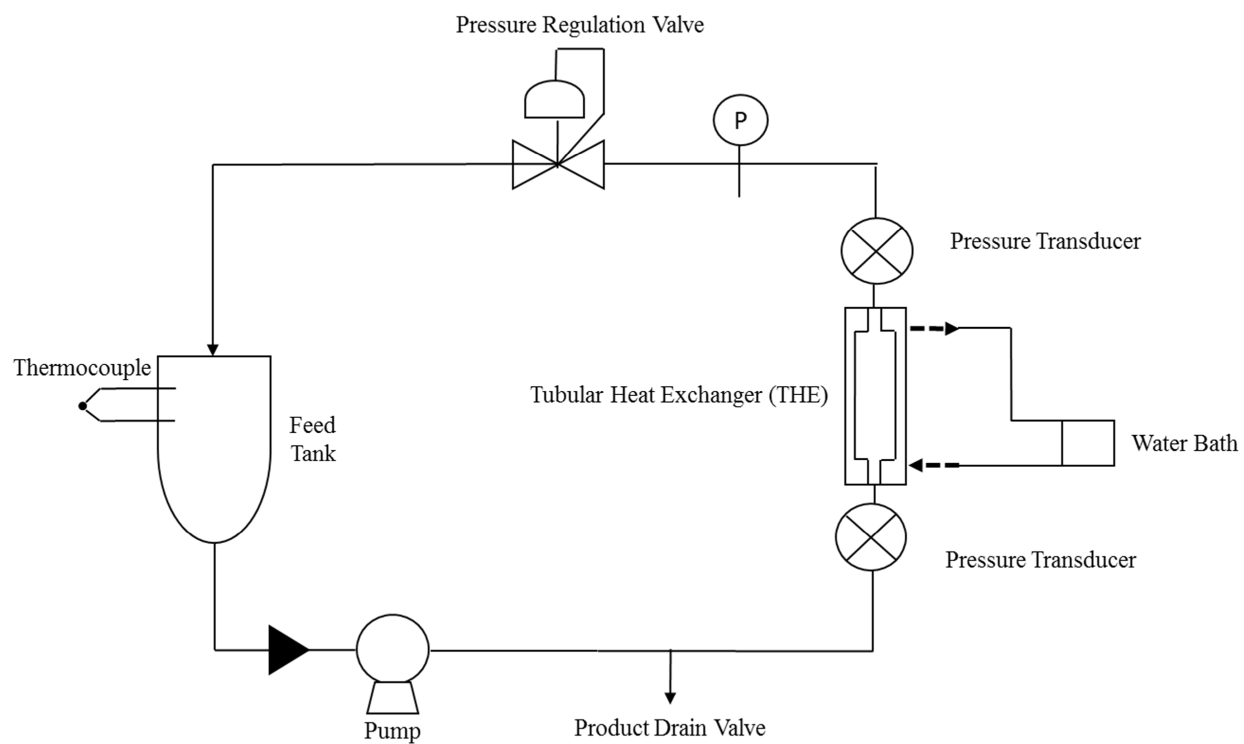

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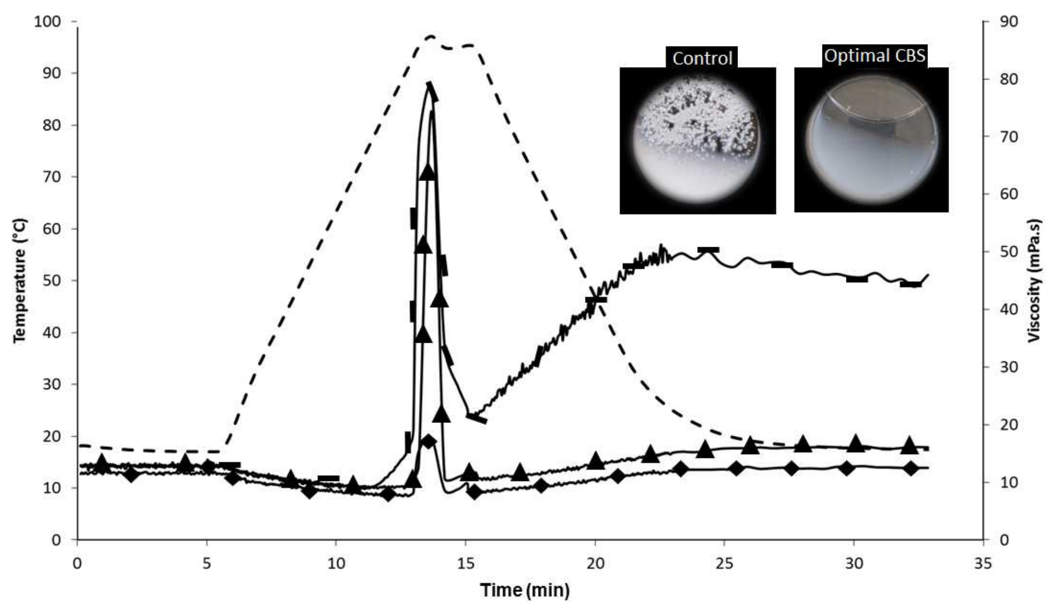
Fig. 2

(A)



(B)

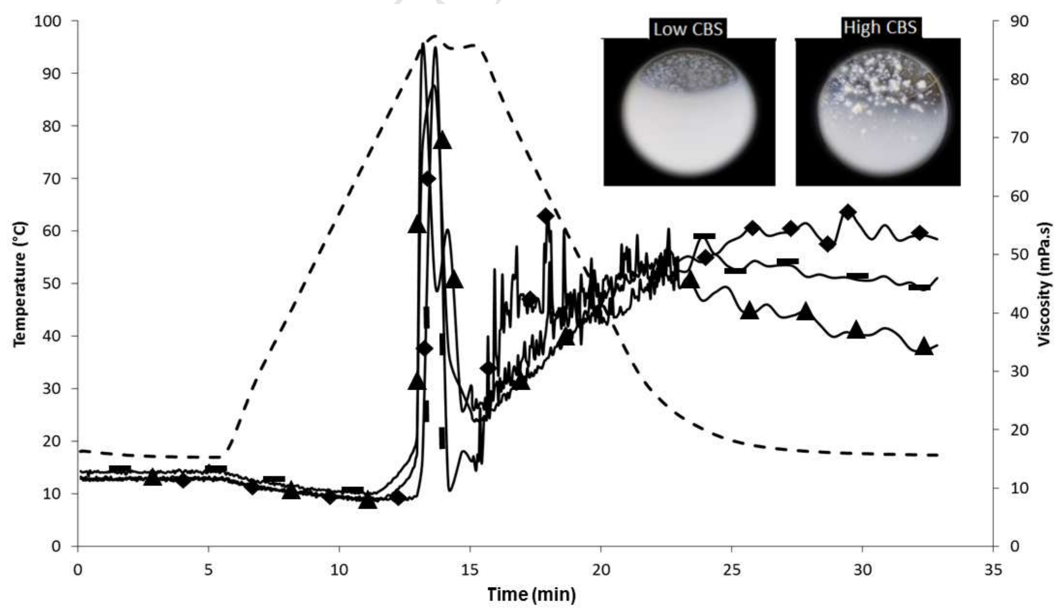


Fig. 3

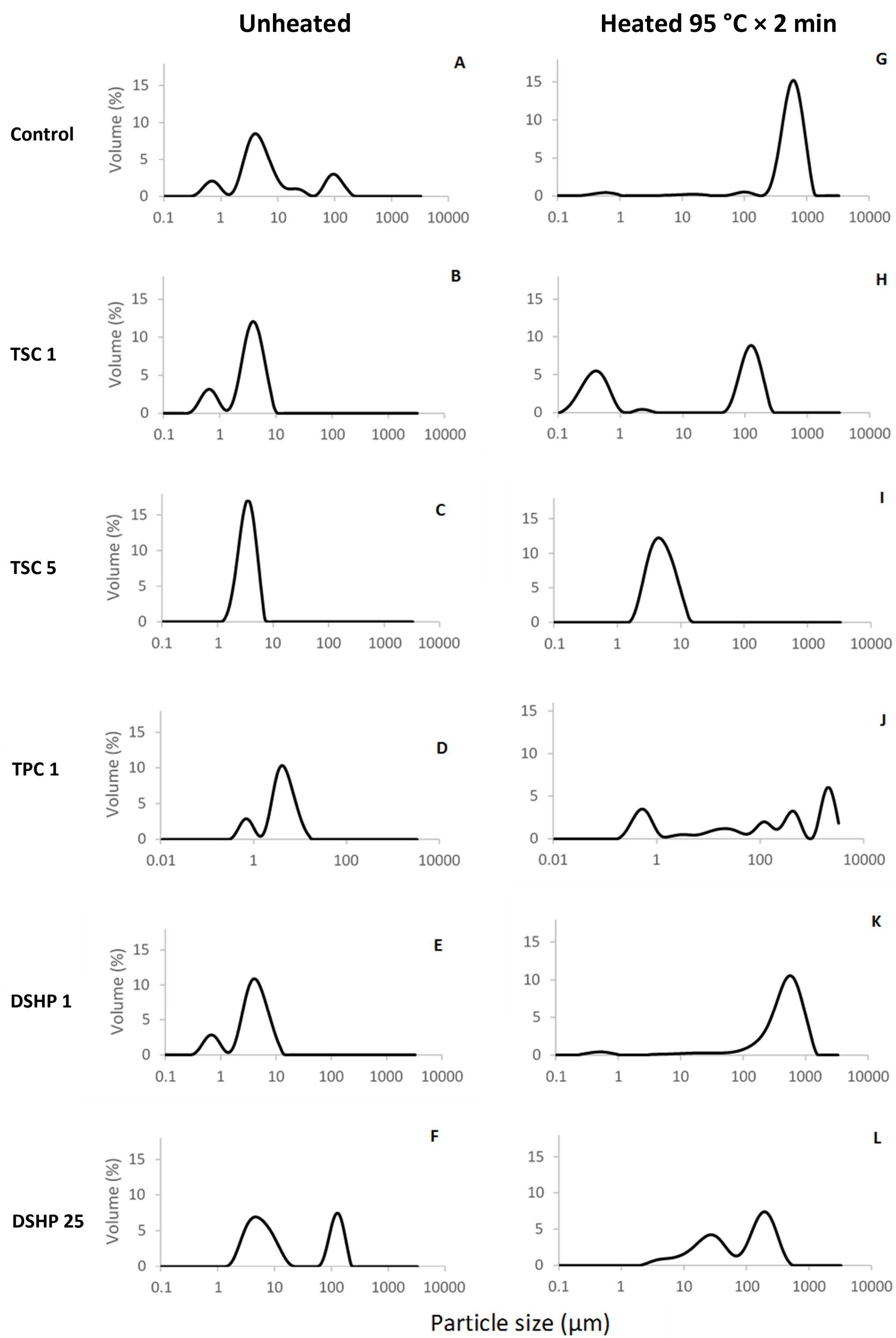


Fig. 4

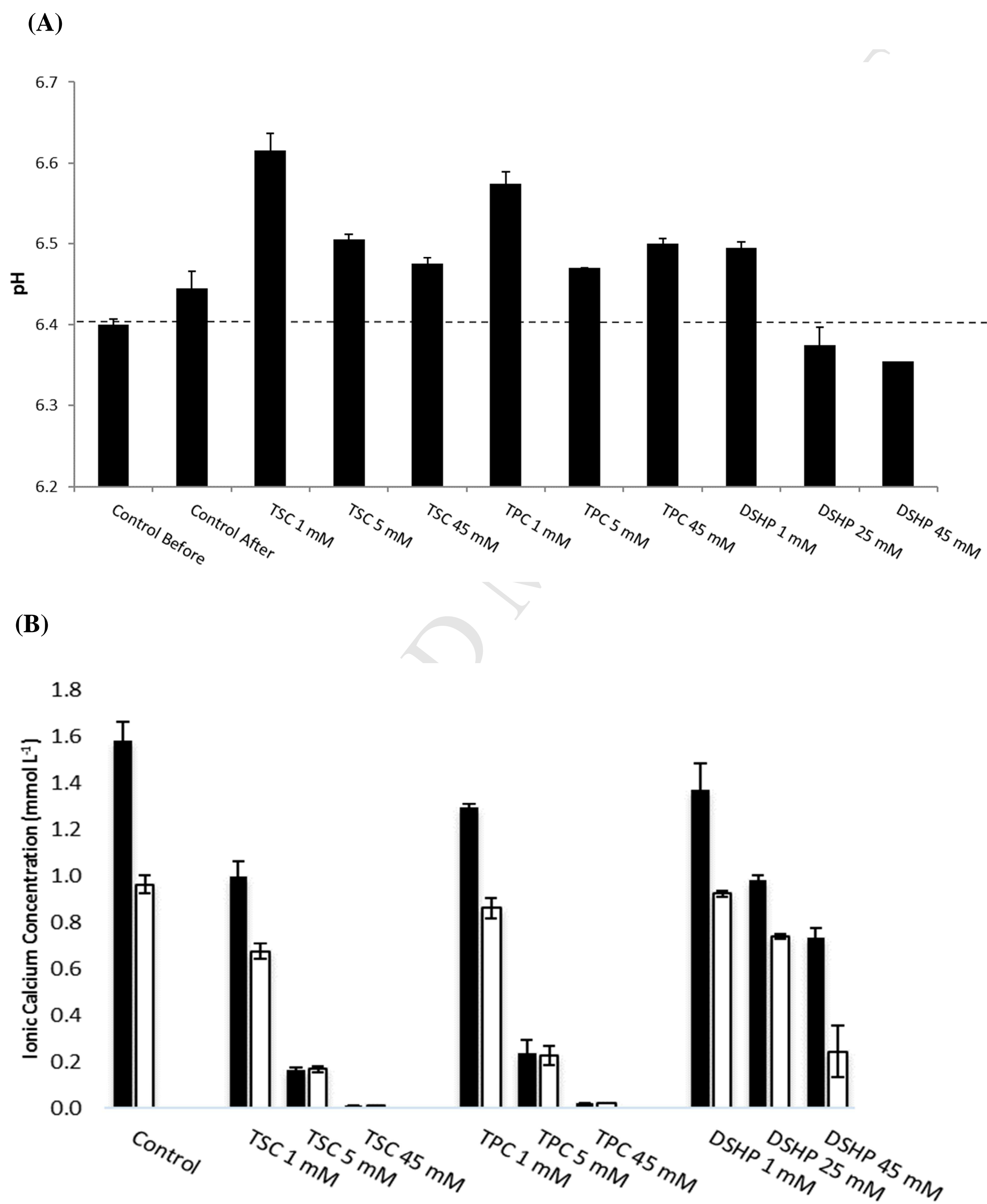
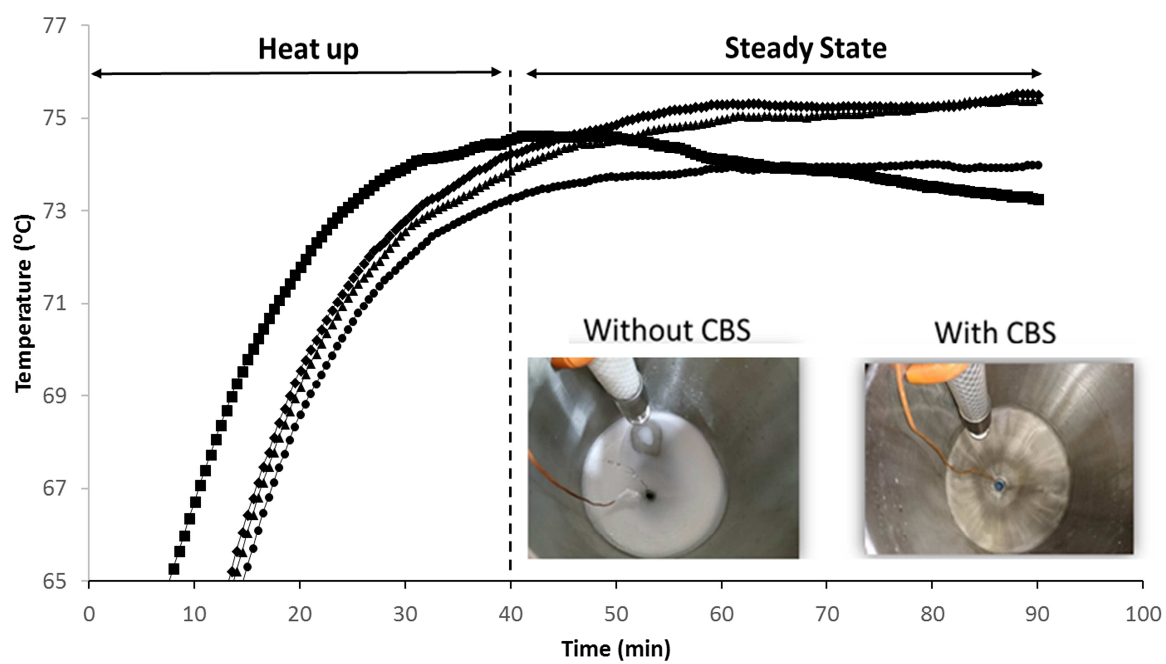


Fig. 5



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