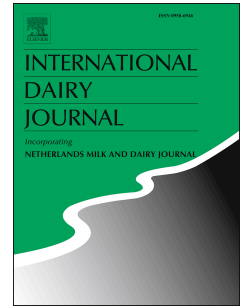


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Influence of sodium hexametaphosphate addition on the functional properties of milk protein concentrate solutions containing transglutaminase cross-linked proteins

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1 **Influence of sodium hexametaphosphate addition on the functional properties of milk**  
2 **protein concentrate solutions containing transglutaminase cross-linked proteins**

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26 ABSTRACT

27

28 The functional properties of milk protein concentrate (MPC) powders are often hindered by  
29 their poor solubility. Calcium chelating salts have been shown to improve powder solubility,  
30 but generally their action contributes to higher viscosity due to disintegration of casein  
31 micelles and higher levels of serum-phase calcium. To help mitigate increases in viscosity  
32 associated with calcium chelation, transglutaminase (TGase), an enzyme that covalently  
33 crosslinks protein, was employed in an effort to stabilise the casein micelle structure. Sodium  
34 hexametaphosphate (SHMP) was added to control (C-MPC) and TGase crosslinked MPC  
35 (TG-MPC) dispersions at concentrations of 5, 12.5 and 25 mM prior to analysis. TG-MPC  
36 dispersions had lower viscosity than C-MPC dispersions across all SHMP concentrations  
37 studied. Crosslinking limited micelle dissociation on SHMP addition and led to greater  
38 retention of the white colour of the protein dispersions, while the turbidity of C-MPC  
39 dispersions decreased with increasing SHMP addition.

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41

## 42 1. Introduction

43

44 High protein dairy based powders, such as milk protein concentrate (MPC), milk  
45 protein isolate, micellar casein isolate and whey protein isolate, are increasingly being used  
46 as ingredients in value-added dairy products such as beverages, yoghurts and infant formulae.  
47 MPC powders are manufactured from skim milk using ultrafiltration, followed by  
48 diafiltration and possible evaporation, prior to spray drying to create high protein powders  
49 that are transported globally (Mistry, 2002; Sikand, Tong, Roy, Rodriguez-Saona, & Murray,  
50 2011).

51 One of the greatest challenges encountered during the processing of milk protein  
52 concentrates is the high viscosity after ultrafiltration and evaporation. This high viscosity can  
53 be caused by the concentration of proteins and an increase in bound moisture due to  
54 denaturation/aggregation of protein during heat treatment (Henriques, Gomes, & Pereira,  
55 2017). In a study by Ho et al. (2018), it was shown that high heat treatment temperatures  
56 resulted in a significant ( $P < 0.05$ ) increase in viscosity from 36.3 to 74.8 mPa s.  
57 Consequently, such high protein systems are typically evaporated to relatively low total  
58 solids content, compared with skim milk, prior to drying (Bienvenue, Jiménez-Flores, &  
59 Singh, 2003; Rupp, Molitor, & Lucey, 2018; Vélez-Ruiz & Barbosa-Cánovas, 1998).

60 A common challenge in using MPC powders is their poor rehydration properties,  
61 which is primarily due to the close proximity of protein molecules and the hydrophobic  
62 nature of the casein protein constituent (Crowley, Desautel, Gazi, Kelly, Huppertz, &  
63 O'Mahony, 2015; de Kruif, Huppertz, Urban, & Petukhov, 2012; Holt, Carver, Ecroyd, &  
64 Thorn, 2013; Horne, 2006; Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2010). Several  
65 different formulation and technological approaches have been investigated to improve  
66 rehydration of MPCs, such as ultrasonication of liquid MPC, calcium depletion using ion

67 exchange and addition of calcium chelating agents (Bhaskar, Singh, & Blazey, 2007;  
68 McCarthy, Kelly, Maher, & Fenelon, 2014; McCarthy, Power, Wijayanti, Kelly, Mao, &  
69 Fenelon, 2017).

70         The use of calcium chelating salts has been shown to improve the dissolution of MPC  
71 when added during the rehydration process (McCarthy et al., 2017); however, their addition  
72 to MPC systems can further contribute to viscosity, resulting in processing challenges during  
73 evaporation, spray drying or in end use applications. Calcium chelators work by sequestering  
74 calcium from the aqueous phase, causing a change in the electrostatic environment and  
75 depletion of calcium from within the casein micelle, through alteration of the calcium  
76 equilibrium. Calcium depletion causes increased hydration and subsequent swelling of casein  
77 micelles due to reduced structural rigidity (de Kort, Minor, Snoeren, van Hooijdonk, & van  
78 der Linden, 2011; McCarthy et al., 2017; Omoarukhe, On-Nom, Grandison, & Lewis, 2010;  
79 Power, Fenelon, O'Mahony, & McCarthy, 2019). Polyphosphate-based chelators, such as  
80 sodium hexametaphosphate (SHMP), have multiple calcium-binding sites, and therefore can  
81 simultaneously bind calcium in the serum phase and calcium from colloidal calcium  
82 phosphate (CCP) nano clusters within casein micelles. Through interactions with colloidal  
83 calcium, SHMP can also crosslink caseins via calcium phosphate complexes, thereby further  
84 increasing viscosity of MPC dispersions (De Kort, Minor, Snoeren, Van Hooijdonk, & Van  
85 Der Linden, 2009, 2011; Lucey & Horne, 2018; Mizuno & Lucey, 2007).

86         The enzyme transglutaminase (TGase) can be used to stabilise the casein micelle.  
87 Previous work by O'Sullivan, Kelly, and Fox (2002a,b) showed that TGase alters the heat  
88 stability of milk by crosslinking individual casein proteins and preventing dissociation of  $\kappa$ -  
89 casein from the micelles. Further research by Smiddy, Martin, Kelly, de Kruif, and Huppertz  
90 (2006) and Moon, Hong, Huppertz, Fox, and Kelly (2009) showed that casein micelles  
91 incubated with TGase had increased stability against micellar disruption by urea, sodium

92 dodecyl sulphate or heating in the presence of ethanol, with stability increasing progressively  
93 with incubation time. Therefore, enzymatic crosslinking by TGase could be used to restrict  
94 increases in viscosity caused by greater hydration and micelle swelling in MPC samples  
95 treated with calcium chelators and also prevent destabilisation of the casein micelle. Thus,  
96 this study aimed to control micelle stability and maintain a lower viscosity of MPC  
97 dispersions in the presence calcium chelating agents by prior enzymatic crosslinking of  
98 casein proteins.

99

## 100 **2. Materials and methods**

101

### 102 *2.1. Materials*

103

104 Milk protein concentrate (MPC) powder (casein:whey protein ratio of 81:19) was  
105 obtained from a local dairy ingredient manufacturer and had protein, moisture, fat, lactose  
106 and ash content of 81.4% (w/w), 4.30% (w/w), 1.40% (w/w), 5.1% (w/w) and 7.8% (w/w),  
107 respectively. Transglutaminase enzyme preparations (Activa MP, Ajinomoto enzyme  
108 preparations) were sourced from Healy Group (Cookstown Industrial Estate, Tallaght, Co.  
109 Dublin, Ireland). Sodium hexametaphosphate (CAS number: 68915-31-1) was obtained from  
110 Sigma Aldrich (Vale Rd, Ballyraine Lower, Arklow, Co. Wicklow, Ireland).

111

### 112 *2.2. Rehydration of milk protein concentrate powder and crosslinking of casein proteins*

113

114 MPC powder was rehydrated (250 g sample at 10%, w/w, protein) as per the method  
115 outlined by Power et al. (2019). Sodium azide (0.02%, w/w) was added to MPC dispersions  
116 to prevent microbial growth. Proteins were covalently cross-linked using the enzyme

117 transglutaminase (TGase) as described previously by Huppertz and de Kruif (2008). MPC  
118 dispersions (10%, w/w) were preheated to 30 °C and incubated with Activia TGase (0.5 g L<sup>-1</sup>)  
119 <sup>1</sup>) for 24 h at pH 6.5. Following incubation, the enzyme was inactivated by heating at 80 °C  
120 for 5 min. A control dispersion was prepared and treated using the same procedure without  
121 the addition of TGase enzyme. Control MPC and enzymatically-crosslinked MPC dispersions  
122 are abbreviated to C-MPC and TG-MPC, respectively.

123

### 124 2.3. *Rheological analysis*

125

#### 126 2.3.1. *Viscosity of milk protein concentrate dispersions as a function of temperature*

127 C-MPC and TG-MPC were divided into 50 mL aliquots and stored at 4, 20, 30 and 50  
128 °C for 3 h prior to rheological analysis. Dispersions were analysed using a controlled-stress  
129 rheometer (AR-G2 Rheometer, TA Instruments, Crawley, UK) equipped with a concentric  
130 cylinder geometry. Sample aliquots were initially conditioned at a temperature of 4, 20, 30 or  
131 50 °C and pre-sheared at 100 s<sup>-1</sup> for 10 s, followed by a peak hold step at a shear rate of 300  
132 s<sup>-1</sup> for 5 min.

133

#### 134 2.3.2. *Viscosity of milk protein concentrate dispersions with sodium hexametaphosphate* 135 *addition*

136 Sodium hexametaphosphate (SHMP) was dissolved in 1 mL of water and the pH  
137 adjusted to 6.5 prior to addition to C-MPC and TG-MPC dispersions (17 mL; 10%, w/w,  
138 protein) to give final SHMP concentrations of 5, 12.5 or 25 mM. Following SHMP addition,  
139 samples were inverted ten times to ensure homogeneity prior to analysis using a controlled  
140 stress rheometer (AR-G2 Rheometer, TA Instruments, Crawley, UK) equipped with a  
141 concentric cylinder geometry. Rheological analysis consisted of a conditioning step

142 performed at 20 °C and a pre-shear at 100 s<sup>-1</sup> for 10 s, followed by a peak hold step at a shear  
143 rate of 100 s<sup>-1</sup> for 2 h.

144

145 *2.4. Particle size distribution of milk protein concentrate dispersions as a function of*  
146 *temperature*

147

148 Particle size measurements were carried out using a Zetasizer nano (Malvern  
149 Instruments, Worcestershire, UK) on both TG-MPC and C-MPC dispersions as a function of  
150 temperature (i.e., 4, 20, 30 or 50 °C) after a 3 h storage period. Dispersions were diluted 1:50  
151 with tempered deionised water prior to analysis. Sample analysis parameters were set at a  
152 dispersant refractive index (RI) of 1.330 and viscosity of 0.8872 cp. The viscosity of  
153 dispersions was measured in disposable cuvettes, and the samples were characterised as  
154 protein using an RI of 1.45 and absorption value of 0.001. Experiments were carried out in  
155 triplicate with a backscattering angle of 173°.

156 Size measurements were also carried out on C-MPC and TG-MPC dispersions with  
157 added SHMP at concentrations of 0, 5, 12.5 and 25 mM using the Zetasizer nano as described  
158 above. All measurements were carried out 1 h after SHMP addition at 20 °C.

159

160 *2.5. Zeta-potential analysis of milk protein concentrate dispersions*

161

162 The  $\zeta$ -potential of C-MPC and TG-MPC dispersions was measured as a function of  
163 pH using a Zetasizer (Malvern Instruments, Worcestershire, UK). Samples were diluted 1:10  
164 using deionised water at 22 °C prior to pH adjustment using concentrated hydrochloric acid  
165 or sodium hydroxide. Zeta potential analysis was performed using water as the dispersant,  
166 with an RI of 1.330 and viscosity of 0.8872 cp. Dispersions were measured using disposable

167 folding capillary cells (DTSI060/DTSI061). The protein was characterised using an RI of  
168 1.45 and absorption value of 0.001. Measurements were performed in triplicate at 25 °C  
169 using the Smoluchowski model (Smoluchowski, 1917).

170

171 *2.6. Colour analysis of control and cross-linked protein concentrate dispersions as a*  
172 *function of sodium hexametaphosphate concentration*

173

174 The colour of C-MPC and TG-MPC dispersions containing 0, 5, 12.5 and 25 mM  
175 SHMP were measured using a Minolta Chroma Meter CR-400 colorimeter (Minolta Ltd.,  
176 Milton Keynes, UK). Data was calculated using three parameters  $L^*$ ,  $a^*$  and  $b^*$  to describe the  
177 colour spectrum of a sample within the three-dimensional visible colour range. The  
178 colorimeter was calibrated against a white standard prior to analysis. Measurements were  
179 taken three times and the mean calculated. Measurement data was displayed as  $L^*$  that  
180 represents a scale from black (0) to white (100),  $a^*$  that represents the green-red spectrum  
181 with a range from -60 (green) to +60 (red) and  $b^*$  that represents the blue-yellow spectrum,  
182 ranging from -60 (blue) to +60 (yellow), respectively (Mohammadi, Rafiee, Emam-Djomeh,  
183 & Keyhani, 2008).

184 Total colour difference ( $\Delta E$ ) was calculated using Equation 1 where  $L_o$ ,  $a_o$  and  $b_o$  refer  
185 to the colour of C-MPC and TG-MPC dispersions without SHMP addition, while  $L$ ,  $a$  and  $b$   
186 denote the respective colour parameters of samples containing SHMP.

$$187 \Delta E = \sqrt{(L_o - L)^2 + (a_o - a)^2 + (b_o - b)^2} \quad (\text{Eq.1})$$

188

189 *2.7. Statistical data analysis*

190

191 All trials and measurements were carried out in triplicate. Rheological measurements  
192 were analysed using a paired T-test with a 95% confidence interval. Particle size and colour  
193 data was statistically analysed using one-way analysis of variance (ANOVA), with post hoc  
194 Tukey analysis. The level of significance was considered as  $P < 0.05$ . All statistical analysis  
195 was carried out using Minitab 17 (Minitab Inc, Coventry, United Kingdom).

196

### 197 3. Results and discussion

198

#### 199 3.1. Rheological and particle size analysis of milk protein concentrate dispersions

200

201 The viscosity of C-MPC and TG-MPC dispersions decreased with increasing  
202 temperature. For example, the viscosity of C-MPC was significantly ( $P < 0.05$ ) lower at 20  
203 °C (11 mPa s) than at 4 °C (37 mPa s) (Fig. 1). The viscosity of TG-MPC dispersions showed  
204 a similar trend (i.e., 15 mPa s at 4 °C and 8 mPa s at 20 °C; Fig. 1), with the viscosity of C-  
205 MPC and TG-MPC dispersions decreasing progressively with increasing temperature;  
206 however, there was no significant ( $P > 0.05$ ) difference in viscosity between non-crosslinked  
207 and crosslinked protein dispersions at 30 or 50 °C. A previous study by Ho et al. (2018)  
208 showed similar results to the present study where the viscosity of MPC increased with  
209 decreasing temperature from 55 to 25 °C. This decrease in viscosity could be attributed to a  
210 higher degree of flexibility within the casein micelles and reduced interactions between  
211 casein micelles, due to a decrease in intra- and intermolecular hydrophobic interactions,  
212 respectively. This is in agreement with particle size distribution profiles shown in Fig. 2,  
213 whereby the z-average diameter for C-MPC samples decreased with increasing temperature  
214 from 163 nm at 4 °C to 156 nm at 50 °C. Weakening of hydrophobic interactions within the

215 casein micelle, results in the release of  $\beta$ -casein from the casein micelle into the serum phase  
216 at low temperature ( $< 20\text{ }^{\circ}\text{C}$ ), leading to increased micelle hydration and size.

217         Conversely, particle size values were lower for TG-MPC dispersions at  $4\text{ }^{\circ}\text{C}$  (158 nm)  
218 than at  $50\text{ }^{\circ}\text{C}$  (170 nm; Fig. 2). The contrasting trend observed for TG-MPC dispersions  
219 compared with C-MPC dispersions could potentially be due to the covalent bond network  
220 created by TGase-induced crosslinking of casein proteins restricting free movement of  
221 proteins (i.e.,  $\beta$ -casein) within and out of the casein micelle at  $4^{\circ}\text{C}$ . Therefore, while  
222 hydrophobic bonds are weaker at  $4\text{ }^{\circ}\text{C}$ , swelling is prevented due to the more rigid cross-  
223 linked protein structure in the TGase treated samples. Casein proteins, both in micellar and  
224 non-micellar form, are the primary substrate for enzymatic crosslinking, which is due to the  
225 abundance of glutamic acid within casein. TGase catalyses cross-linking of peptide chains  
226 through the formation of an isopeptide bond between the  $\gamma$ -carboxyamide group of glutamine  
227 side chains and an amine donor of neighbouring lysine or glutamine residues depending on  
228 steric location (Jaros, Partschefeld, Henle, & Rohm, 2006; Moon et al., 2009; O'Sullivan et  
229 al., 2002a,b). Ercili-Cura et al. (2013) reported that gels produced from TGase modified milk  
230 had a significantly higher water holding capacity as a result of restricted particle movement  
231 due to crosslinking. Prior incubation of milk with TGase resulted in gels consisting of more  
232 fixed networks of small aggregates with defined pore sizes; hence preventing network  
233 contraction/rearrangement and subsequent syneresis of water. Potentially the high water  
234 holding capacity of TG-MPC dispersions could result in larger particle size values as a  
235 consequence of casein micelle swelling at increased temperatures (i.e.  $50\text{ }^{\circ}\text{C}$ : Fig. 2D).

236

237 *3.2. Zeta-potential of milk protein concentrate dispersions*

238

239 The  $\zeta$ -potential of C-MPC and TG-MPC dispersions as a function of pH are shown in  
240 Fig. 3. At an initial pH of 6.7, C-MPC had a  $\zeta$ -potential of  $-18.8$  mV while TG-MPC had a  $\zeta$ -  
241 potential of  $-20.7$  mV (Fig. 3). These values are in line with previous work carried out on  
242 skim milk at pH 6.7, which had a  $\zeta$ -potential of  $-18$  mV (Wade, Beattie, Rowlands, &  
243 Augustin, 1996). As expected, the  $\zeta$ -potential of both C-MPC and TG-MPC dispersions  
244 became less negative with decreasing pH. Nogueira et al. (2019) reported similar results with  
245  $\zeta$ -potential values of approximately  $-20$  mV at pH 6.0 for a cross-linked micellar casein  
246 system. However, in the present study, the  $\zeta$ -potential at pH 5.5 for C-MPC and TG-MPC  
247 was  $-14.3$  and  $-13.9$  mV, respectively, compared with Nogueira et al. (2018) who reported a  
248  $\zeta$ -potential of  $-18$  mV at pH 5, while at pH less than 5.5 in the present study, the  $\zeta$ -potential  
249 was not measured due to extensive precipitation of casein. The fact that there were no  
250 significant differences in  $\zeta$ -potential between C-MPC and TG-MPC dispersions showed that  
251 crosslinking did not alter the surface charge of the casein micelles. This is in line with  
252 previous work carried out by de Kruif, Tuinier, Holt, Timmins, and Rollema (2002) who  
253 showed, using small-angle neutron scattering (SANS), that crosslinking of casein micelles  
254 caused little or no restructuring of the casein micelle other than the formation of covalent  
255 linkages.

256

257 *3.3. Particle size and colour analysis of milk protein concentrate dispersions containing*  
258 *sodium hexametaphosphate*

259

260 C-MPC dispersions without (0 mM) and with 5 mM SHMP addition displayed narrow  
261 monomodal size distribution profiles, with particle size ranging from 68.1 to 459 nm (Fig.  
262 4A). However, C-MPC dispersions containing 12.5 and 25 mM SHMP were significantly  
263 different, with a shift in profile towards a broader particle size distribution (Fig. 4A). The

264 effect of SHMP addition on the size distribution profiles of MPC dispersions was also  
265 highlighted by the significant ( $P < 0.05$ ) changes in polydispersity index (PdI) (Table 1), with  
266 PdI values of C-MPC dispersions increasing (0.09–0.41) with increasing SHMP content  
267 (Table 1). Particle size results for C-MPC dispersions correlated well with colour analysis  
268 (Table 2), with C-MPC dispersions containing 0 and 5 mM being relatively similar in terms of  
269  $L^*$ -values (82.7 and 80.2, respectively) but with some differences observed in  $b^*$ -values, in  
270 conjunction with a  $\Delta E$  value (i.e., 3.10; Table 2) denoting a visible change. A significantly ( $P$   
271  $< 0.05$ ) lower  $L^*$ -value (denoting whiteness) was observed for C-MPC dispersions containing  
272 12.5 and 25 mM SHMP (48.3 and 46.0, respectively), with respective  $\Delta E$  values of 34.5 and  
273 37.0 (Table 2; Fig. 4A inset). Considered collectively, these data provide evidence for the  
274 dissociation of casein micelles into primary casein particles (De Kort et al., 2009, 2011;  
275 Panouillé, Benyahia, Durand, & Nicolai, 2005; Pitkowski, Nicolai, & Durand, 2008). Strong  
276 polyphosphate-based calcium chelators, such as SHMP, cause partial disintegration of casein  
277 micelles by the depletion of calcium from the casein micelle, resulting in partial collapse of  
278 the micelle, releasing individual casein proteins/particles into the serum phase.

279 No differences were observed in particle size distribution profiles for TG-MPC  
280 dispersions containing 5 and 12.5 mM SHMP compared with the control. Previous studies  
281 (Myllärinen, Buchert, & Autio, 2007; O'Sullivan, Kelly, & Fox, 2002) showed similar results  
282 to those found in the current study, with TGase-treated casein micelles having increased  
283 resistance to dissociation during calcium depletion. Only when 25 mM SHMP was added to  
284 TG-MPC dispersions was a broadening of particle size distribution profile observed, with a  
285 primary peak between 78.8 and 825 nm, indicating increased casein micelle swelling and  
286 hydration, with a secondary smaller peak between 28.2 and 78.8 nm, indicating the presence  
287 of smaller casein micelle fragments (Fig. 4B). These primary casein particles have been  
288 shown to have a diameter of approximately 20 nm (De Kort et al., 2009, 2011; Huppertz et al.,

289 2017; Panouillé et al., 2005; Pitkowski et al., 2008). This was also observed in PDI values for  
290 TG-MPC samples, with no significant ( $P > 0.05$ ) difference in dispersions between 0 and  
291 12.5 mM (0.13 to 0.14), while at 25 mM, the PDI value was significantly ( $P < 0.05$ ) higher at  
292 0.24 (Table 1). Similarly, the  $L^*$ -value for TG-MPC dispersions also decreased with  
293 increasing SHMP addition level ( $L^*$ -value decreased from 84.0 to 59.9; Table 2), albeit to a  
294 significantly ( $P < 0.05$ ) lesser extent than observed for C-MPC dispersions. The resistance of  
295 TG-MPC dispersions to disintegration, and retention of whiteness, can be related to the  
296 strong isopeptide bond formed between amino acids during TGase incubation process.  
297 Therefore, casein micelles treated with TGase had improved micelle stability and as a result  
298 retained more light scattering ability in the presence of high concentrations of SHMP (see  
299 Fig. 4B inset).

300

#### 301 3.4. *Viscosity measurements of milk protein concentrate dispersions containing sodium* 302 *hexametaphosphate*

303

304 Viscosity profiles of C-MPC and TG-MPC dispersions with added SHMP at  
305 concentrations of 0, 5, 12.5 and 25 mM are shown in Fig. 5. At 5 mM SHMP addition both C-  
306 MPC and TG-MPC dispersions had higher viscosity with final values of 25 and 29 mPa s,  
307 respectively, compared with the respective samples without SHMP addition. Significantly ( $P$   
308  $< 0.05$ ) higher viscosity was observed in C-MPC dispersions containing 12.5 and 25 mM  
309 SHMP, with final viscosities of 48 to 3217 mPa s and from 72.7 to 3838 mPa s, respectively  
310 (Fig. 5A). This higher viscosity can be attributed to chelation of calcium, which causes  
311 diffusion of calcium from the micelle into the serum phase and a reduction in the proportion  
312 of micellar CCP. The reduction in CCP causes increased electrostatic repulsion between  
313 casein proteins within the micelle, which together with the loss of the CCP-mediated casein

314 protein cross-links, causes reduced structural integrity and increased swelling of the casein  
315 micelles (De Kort et al., 2009, 2011; Holt, 1992).

316 TG-MPC dispersions displayed considerably less change in viscosity with addition of  
317 SHMP (Fig. 5B). At higher addition levels of SHMP (i.e., 12.5 and 25 mM), TG-MPC  
318 dispersions had significantly ( $P < 0.05$ ) lower final viscosity values (162 and 991 mPa s,  
319 respectively), compared with the corresponding C-MPC dispersions (3212 and 3838 mPa s,  
320 respectively). The resistance of TG-MPC to increases in viscosity on the addition of SHMP  
321 can be attributed to the impact of enzymatic crosslinking, which creates a secondary  
322 structural organisation within the casein micelles, conferring increased resistance to micellar  
323 disintegration.

324 TGase-mediated crosslinking could also potentially hinder the access of SHMP to  
325 CCP within the casein micelle. Previous work carried out by Power et al. (2019) showed that  
326 a change in  $^{31}$ P phosphate nuclear magnetic resonance signal occurred upon the addition of  
327 SHMP to reconstituted MPC dispersions, indicative of a change in the structure of phosphate  
328 prompted by an interaction between SHMP and phosphate-bound calcium associated with the  
329 casein micelle. Chelation increases exposure of negatively-charged phosphate residues  
330 through depletion of bound calcium, hence changing the charge on the phosphate residues  
331 and its associated resonance signal (Holt, 1997; Walstra, 1990). Therefore, TGase-mediated  
332 crosslinking may restrict the ability of SHMP to chelate calcium bound to phosphate residues  
333 along the casein structure, creating a calcium-casein phosphate complex.

334

#### 335 **4. Conclusion**

336

337 The chelation of calcium in milk protein concentrate dispersions significantly  
338 modifies the structural integrity of casein micelles, leading to increased viscosity. In this

339 study, the use of transglutaminase to cross-link casein micelles prior to addition of sodium  
340 hexametaphosphate greatly reduced this viscosity development, with the effect being most  
341 pronounced at low temperature. Enzymatically crosslinking casein micelles also helped  
342 maintain the natural white colour of MPC even after SHMP addition. This may also be useful  
343 in other studies which use strategies such as ion-exchange to reduce the calcium content of  
344 micellar casein ingredients. Overall, this study provided new knowledge relating to the  
345 factors responsible for increased viscosity during calcium chelation, mainly casein micelle  
346 swelling and micelle dissociation, and demonstrated that enzymatic crosslinking is effective  
347 in controlling viscosity development in MPC systems with added calcium chelating salts.

348

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350

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353

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1 **Figure legends**

2

3 **Fig. 1.** Apparent viscosity of control (■) and transglutaminase cross-linked (□) milk protein  
4 concentrate dispersions (10%, w/w, protein) at 4, 20, 30 or 50 °C, measured at a shear rate of  
5  $100 \text{ s}^{-1}$ . Values presented are the mean values  $\pm$  SD.

6

7 **Fig. 2.** Particle size distribution profiles of control (—) and transglutaminase cross-linked (---  
8 -) milk protein concentrate dispersions (10%, w/w, protein) measured at temperatures of: A, 4  
9 °C; B, 20 °C; C, 30 °C; D, 50 °C.

10

11 **Fig. 3.** Zeta-potential of control (■) and transglutaminase cross-linked (◆) milk protein  
12 concentrate dispersions (10%, w/w, protein), as a function of pH. Values presented are the  
13 mean values  $\pm$  SD.

14

15 **Fig. 4.** Particle size distribution profiles of control (A) and transglutaminase cross-linked (B)  
16 milk protein concentrate dispersions (10%, w/w, protein) containing 0 (—), 5 (— —), 12.5  
17 (---) and 25 (.....) mM sodium hexametaphosphate. Insets: photographic image of control  
18 and transglutaminase cross-linked milk protein concentrate dispersions containing 0 (i), 5 (ii),  
19 12.5 (iii) or 25 (iv) mM sodium hexametaphosphate.

20

21 **Fig. 5.** Viscosity profiles of control (A) and transglutaminase cross-linked (B) milk protein  
22 concentrate dispersions (10%, w/w, protein) containing 0 (—), 5 (.....), 12.5 (---) and 25  
23 (---·) mM sodium hexametaphosphate, measured at a shear rate of  $100 \text{ s}^{-1}$  at 20 °C.

**Table 1**

Particle size distribution parameters for control and transglutaminase cross-linked milk protein concentrate dispersions with added sodium hexametaphosphate. <sup>a</sup>

SHMP concentration (mM)	C-MPC		TG-MPC	
	Z-average (nm)	PdI (-)	Z-average (nm)	PdI (-)
0	180	0.09	182	0.13
5	180	0.10	182	0.12
12.5	138	0.34	200	0.14
25	988	0.41	211	0.24

<sup>a</sup> Abbreviations are: C-MPC, control milk protein concentrate; TG-MPC, transglutaminase cross-linked milk protein concentrate; SHMP, sodium hexametaphosphate; Z-average, intensity weighted mean hydrodynamic size of particles measured by dynamic light scattering; PdI, polydispersity index.

**Table 2**

Colour chromaticity co-ordinates of control and transglutaminase cross-linked milk protein concentrate dispersions with added sodium hexametaphosphate. <sup>a</sup>

SHMP concentration (mM)	C-MPC				TG-MPC			
	$L^*$	$a^*$	$b^*$	$\Delta E$	$L^*$	$a^*$	$b^*$	$\Delta E$
0	82.7 <sup>a</sup>	-4.20 <sup>c</sup>	2.06 <sup>b</sup>	-	84.0 <sup>a</sup>	-4.14 <sup>a</sup>	1.79 <sup>a</sup>	-
5	80.2 <sup>a</sup>	-4.67 <sup>c</sup>	0.29 <sup>d</sup>	3.10 <sup>a</sup>	82.5 <sup>a</sup>	-4.63 <sup>a</sup>	1.04 <sup>b</sup>	1.76 <sup>a</sup>
12.5	48.3 <sup>b</sup>	-1.65 <sup>b</sup>	1.93 <sup>c</sup>	34.5 <sup>b</sup>	66.3 <sup>b</sup>	-5.08 <sup>a</sup>	-4.33 <sup>c</sup>	18.8 <sup>b</sup>
25	46.0 <sup>c</sup>	-0.67 <sup>a</sup>	3.84 <sup>a</sup>	37.0 <sup>c</sup>	59.9 <sup>c</sup>	-4.26 <sup>a</sup>	-4.45 <sup>c</sup>	24.9 <sup>c</sup>

<sup>a</sup> Abbreviations are: C-MPC, control milk protein concentrate; TG-MPC, transglutaminase cross-linked milk protein concentrate; SHMP, sodium hexametaphosphate;  $L^*$ , whiteness;  $a^*$ , green-red spectrum with a range from -60 (green) to +60 (red);  $b^*$ , blue-yellow spectrum with a range from -60 (blue) to +60 (yellow);  $\Delta E$ , total colour difference. Values within a column not sharing a common superscript letter differ significantly ( $P < 0.05$ ).



Figure 1

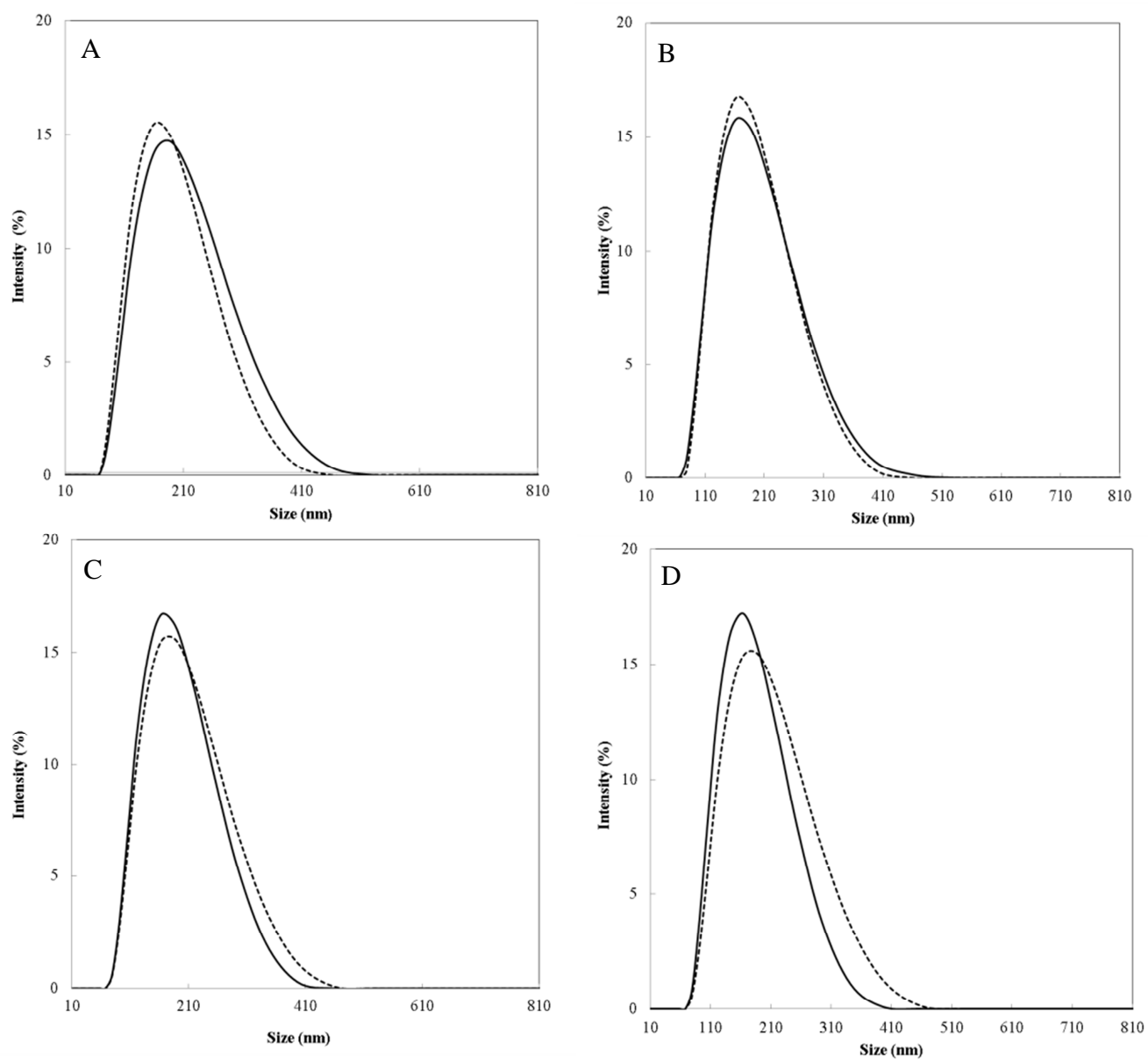


Figure 2

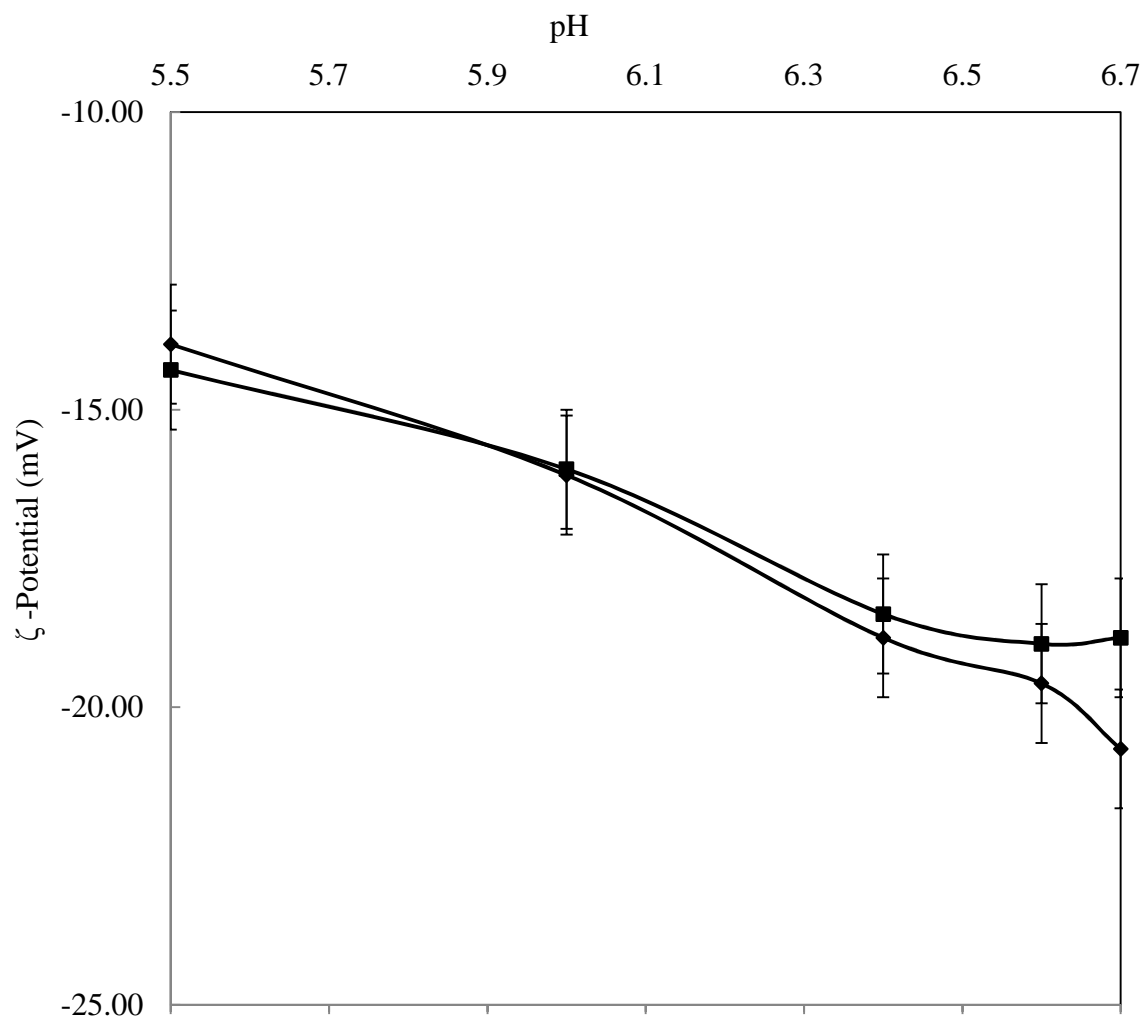


Figure 3

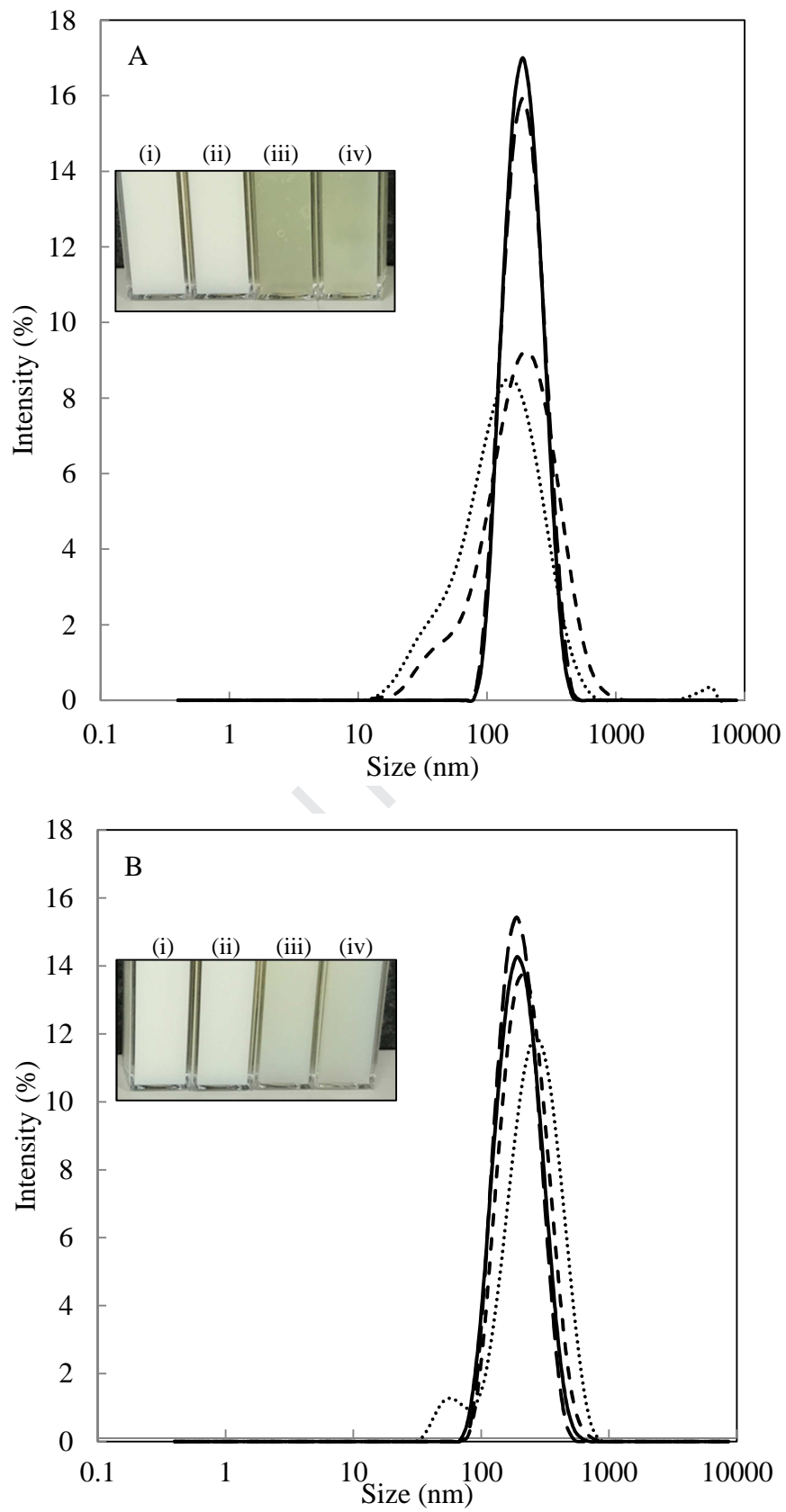


Figure 4

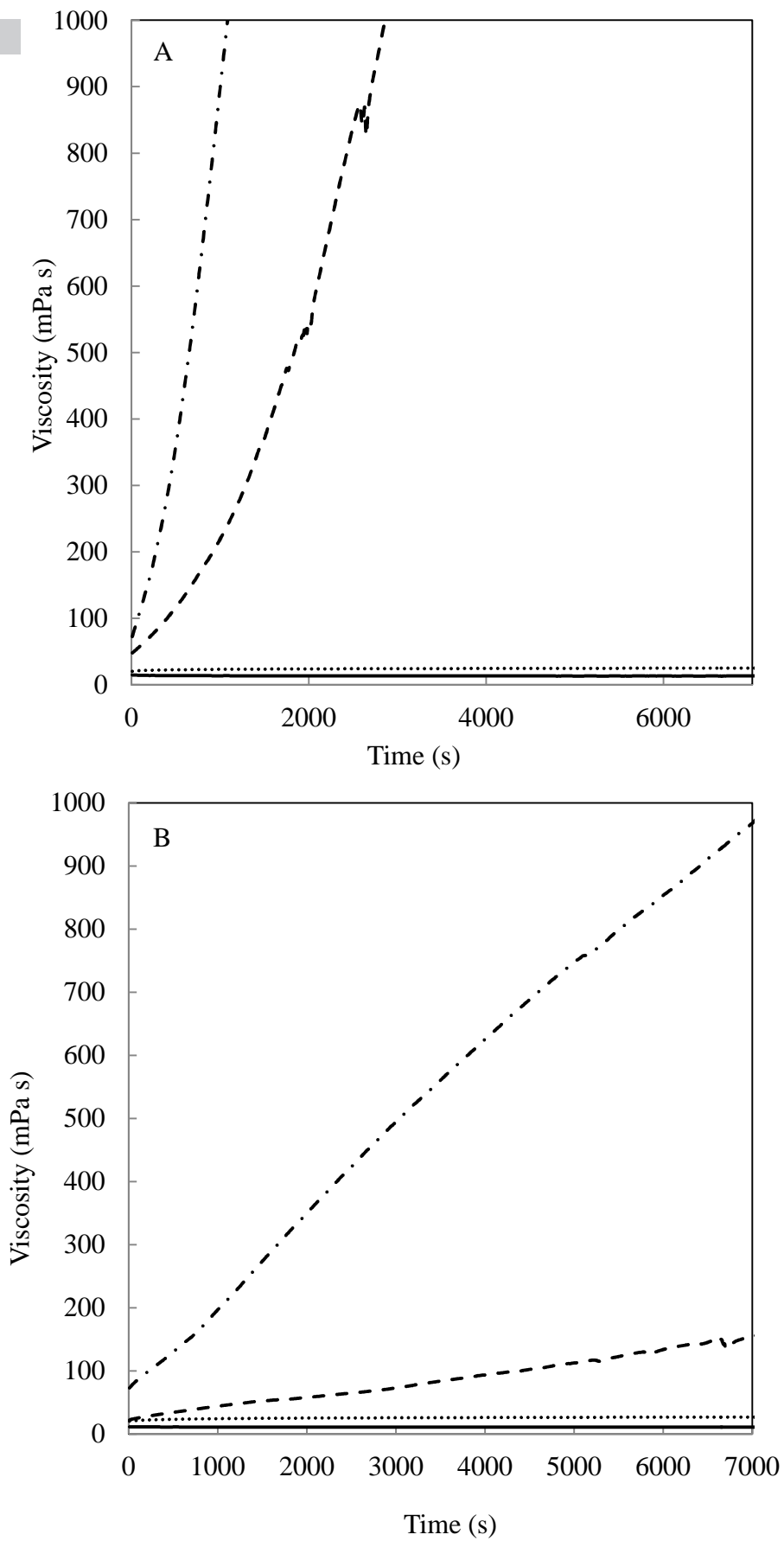


Figure 5