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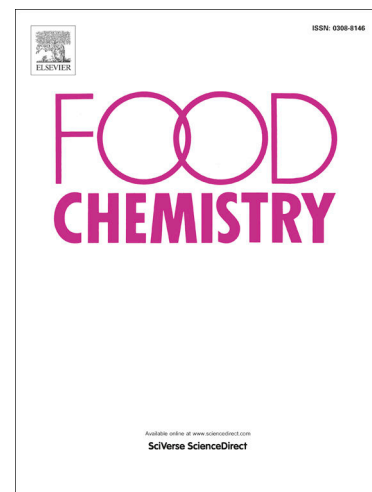
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1 Influence of calcium fortification on physicochemical properties of  
2 whey protein concentrate solutions enriched in  $\alpha$ -lactalbumin

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9 **Abstract**

10 In this study, three whey protein concentrate systems enriched in  $\alpha$ -lactalbumin, produced  
11 using membrane separation (LAC-M), selective precipitation (LAC-P) and ion-exchange  
12 chromatography (LAC-IE), were fortified with calcium chloride ( $\text{CaCl}_2$ ) at 0-5 mM and  
13 changes in physicochemical properties studied. Binding of calcium ( $\text{Ca}^{2+}$ ) occurred for LAC-  
14 P in the range 0.00-2.00 mM, with an affinity constant ( $K_d$ ) of  $1.63 \times 10^{-7}$ , resulting in a  
15 proportion of total protein-bound calcium of 81.8% at 2 mM  $\text{CaCl}_2$ . At 5 mM  $\text{CaCl}_2$ , LAC-P  
16 had volume mean diameter (VMD) of 638 nm, while LAC-M and LAC-IE had VMD of 204  
17 and 3.87 nm, respectively. Changes in physicochemical properties were dependent on the  
18 approach used to enrich  $\alpha$ -lactalbumin and concentrations of other macromolecules (e.g.,  
19 phospholipid). The results obtained in this study provide fundamental insights into the  
20 influence of fortification with soluble calcium salts on the physicochemical stability of next-  
21 generation whey protein ingredients enriched in  $\alpha$ -lac.

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## 34 1. Introduction

35 Nutritional dairy-based products fortified with calcium are widely available; however,  
36 fortification of such products with calcium remains challenging. Bovine milk contains 30  
37 mM total calcium, of which 20 mM is in the colloidal state (i.e., associated with casein  
38 proteins in the micelles) and approximately 10 mM is soluble (in different forms, such as  
39 phosphate and citrate salts), with a subset (typically 1-3 mM) of the soluble fraction being  
40 present in ionic form ( $\text{Ca}^{2+}$ ) (Lewis, 2011). Fortification of nutritional dairy-based products  
41 with soluble calcium salts (e.g., calcium chloride, calcium hydroxide and calcium gluconate)  
42 increases the concentration of  $\text{Ca}^{2+}$ , which can lead to protein instability, with whey proteins  
43 being more susceptible than caseins (Crowley, Kelly, & O'Mahony, 2014). Also, the  
44 contribution of the calcium salt counter-ion (e.g., chloride, phosphate and hydroxide) can  
45 influence the physicochemical properties (e.g., pH, freezing point and buffering capacity) of  
46 calcium-fortified dairy-based nutritional products (Omoarukhe, On-Nom, Grandison, &  
47 Lewis, 2010).

48 Whey proteins generally display good physicochemical stability in solution at pH  
49 values away from their isoelectric point (pI), due to a high charge-to-mass ratio (Foegeding,  
50 Davis, Doucet, & McGuffey, 2002). At the pH of most dairy-based nutritional beverage  
51 products (typical pH 6.5-7.0) whey proteins are negatively charged, primarily due to the  
52 carboxylic acid ( $\text{pK}_a \sim 5.10$ ) residues of the protein. Increasing  $\text{Ca}^{2+}$  level reduces the surface  
53 charge on whey proteins, thereby decreasing the electrostatic repulsion between proteins  
54 (Keowmaneechai & McClements, 2002). This interaction has been reported to be caused by  
55 calcium-mediated bridging between the carboxylic acid groups of aspartic and glutamic acids,  
56 resulting in crosslinking of individual whey protein molecules, leading to aggregation and  
57 potential gel formation (Barbut & Foegeding, 1993).

58 In contrast to these types of interactions with whey protein,  $\text{Ca}^{2+}$  can also increase the  
59 stability of selected proteins if the ions are strongly bound to a specific intramolecular  
60 binding site; this type of interaction is known to occur for the whey protein  $\alpha$ -lactalbumin ( $\alpha$ -  
61 lac), and to a lesser extent for  $\beta$ -lactoglobulin ( $\beta$ -lg) (Jeyarajah & Allen, 1994). The affinity  
62 of  $\alpha$ -lac for  $\text{Ca}^{2+}$  is considerably higher in the apo-state (i.e., calcium-depleted) compared to  
63 the holo-state (i.e., calcium-bound) of the protein. The binding of  $\text{Ca}^{2+}$  by *apo*- $\alpha$ -lac results in  
64 conformational changes to the protein, serving to increase stability of the protein to  
65 denaturation when subjected to thermal treatment (Permyakov & Berliner, 2000a).

66 The most commonly encountered challenges with calcium-fortified whey-based  
67 nutritional products arise from protein aggregation, increased viscosity, gel formation,  
68 fouling and poor heat transfer efficiency (Ju & Kilara, 1998; Khaldi et al., 2018). A number  
69 of strategies have been investigated to overcome these challenges, such as preheating of whey  
70 protein (Joyce, Brodkorb, Kelly, & O'Mahony, 2017), modification of whey protein profile  
71 to increase  $\alpha$ -lac: $\beta$ -lg ratio (Crowley, Dowling, Caldeo, Kelly, & O'Mahony, 2016),  
72 alteration of pH and protein charge (Anema, 2018), and addition of calcium-binding salts to  
73 sequester  $\text{Ca}^{2+}$  (Hebishy, Joubran, Murphy, & O'Mahony, 2019).

74 Whey protein concentrate (WPC) enriched in  $\alpha$ -lac (LAC) is a category of whey-  
75 based ingredient used in the formulation of nutritional dairy-based products such as infant  
76 milk formula, to better match the protein profile of human milk. Such ingredients also have  
77 nutritional applications through the delivery of sufficient levels of tryptophan, which is  
78 essential for serotonin synthesis and thereby beneficial for human wellbeing (e.g., regulation  
79 of circadian rhythm, mood, memory function, and cognitive performance) (Silber & Schmitt,  
80 2010). This type of value-added ingredient can be manufactured using different approaches,  
81 resulting in ingredients with different physicochemical properties (Barone, O'Regan, &  
82 O'Mahony, 2019).

83           The influence of  $\text{Ca}^{2+}$  on the physicochemical and functional properties (e.g., heat  
84 stability, gelation and emulsification) of whey proteins has been most extensively studied  
85 using whey protein ingredients with unaltered protein profile (Keowmaneechai &  
86 McClements, 2002; Kharlamova, Nicolai, & Chassenieux, 2018; Ye & Singh, 2000). In this  
87 study, the influence of fortification of WPC enriched in  $\alpha$ -lac using different technological  
88 approaches, with soluble calcium in the form of  $\text{CaCl}_2$ , on physicochemical (e.g., particle size  
89 distribution and zeta potential), thermodynamic (i.e., Gibbs free energy, enthalpy, entropy  
90 and stoichiometry) and colloidal stability of the systems was investigated. This novel work  
91 will support the development of calcium-fortified whey protein-based beverage systems with  
92 protein profiles tailored to meet specific nutritional requirements.

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## 108 2. Materials and methods

### 109 2.1 Materials

110 Three spray-dried  $\alpha$ -lactalbumin-enriched WPC (LAC) ingredients were obtained  
111 from three different manufacturers across the European Union and United States of America,  
112 manufactured in all cases from sweet whey. LAC-M was manufactured using membrane  
113 filtration of whey to selectively retain higher molecular weight whey proteins (e.g.,  $\beta$ -  
114 lactoglobulin), with  $\alpha$ -lac enriched in the permeate stream. LAC-P was manufactured using  
115 membrane filtration to reduce the levels of low molecular weight, non-protein components  
116 (e.g., lactose and minerals), before selective precipitation of  $\alpha$ -lac by targeted adjustment of  
117 pH, ionic strength and temperature. LAC-IE was manufactured using ion-exchange  
118 chromatography-based separation of  $\alpha$ -lac and  $\beta$ -lg in liquid whey.

119 The protein content determined using the Kjeldahl method (Lynch, & Barbano, 1999)  
120 of LAC-M, LAC-P and LAC-IE powders was 78.8, 78.2 and 92.5% (w/w), respectively. The  
121  $\alpha$ -lac content of LAC-M, LAC-P and LAC-IE powders was 28.4, 24.4 and 73.4% (w/w),  
122 giving  $\alpha$ -lac: $\beta$ -lactoglobulin ( $\beta$ -lg) ratios of 1.72:1, 2.48:1 and 13.3:1, respectively. Regular  
123 whey protein isolate (WPI) and concentrate (WPC) ingredients were used as benchmarks  
124 with 88.1 and 33.3% (w/w) protein, respectively, and  $\alpha$ -lac contents of 20.4 and 4.36%  
125 (w/w), giving  $\alpha$ -lac: $\beta$ -lg ratios of 0.24:1 and 0.28:1, respectively. The  $\alpha$ -lac and  $\beta$ -lg content  
126 was measured by reversed-phase high performance liquid chromatography using the method  
127 described by Jackson et al. (2004). Further information on the composition of these  
128 ingredients is available in Barone, O'Regan, & O'Mahony (2019). The total calcium content  
129 of the ingredients was determined by inductively coupled plasma-mass spectrometry  
130 according to the method of (Herwig, Stephan, Panne, Pritzkow, & Vogl, 2011); WPC, WPI,  
131 LAC-M, LAC-P and LAC-IE had total calcium contents of 704, 82.6, 500, 3.58 and 198  
132 mg/100 g of powder, respectively. The total fat content of the powders was determined using



133 the Röse-Gottlieb method (AOAC, 2006), with WPC, WPI, LAC-M, LAC-P and LAC-IE  
134 having fat contents of 2.45, 0.59, 0.88, 9.32 and 0.36% w/w. A sub-sample of LAC-P was  
135 defatted according to the method described by Castro-Gómez et al. (2014), with some  
136 modifications. Briefly, powder was dispersed (5%, w/v) in a 2:1 dichloromethane/methanol  
137 solvent mixture at 25°C and stirred for 20 min at 750 rpm, with the mixture being held  
138 quiescently for 25 min, after which the clarified organic solvent was decanted and filtered  
139 through Whatman filter paper grade 541 (GE Healthcare, Chicago, IL, USA). The extraction  
140 of fat was carried out three times for the same powder, after which the defatted material was  
141 dried using a laboratory scale Edwards Modulyo F101 freeze drier (Edwards, Crawley, UK).  
142 The fat and protein contents of the defatted variant of LAC-P (LAC-P-D) sample was 0.28  
143 and 87.1% (w/w), respectively. The total phospholipid (PL) content of the original LAC-P  
144 and LAC-P-D was 4.68 and 0.36% (w/w), respectively, as determined according to the  
145 method of Braun, Flück, Cotting, Monard, & Giuffrida (2010) using high performance liquid  
146 chromatography (Agilent 1100, Santa Clara, USA) equipped with an evaporative light  
147 scattering detector at 80°C using a gas flow rate of 1 L/min.

148

## 149 *2.2 Preparation and calcium fortification of whey protein solutions*

150 The protein powders were reconstituted in ultra-pure water to 1% (w/v) protein, using  
151 magnetic stirring at 350 rpm for at least 2 h, followed by holding at 4°C for 18 h with  
152 continued stirring. Prior to analysis, the pH of the protein solutions was adjusted to pH 6.80  
153 using 0.5 M potassium hydroxide or hydrochloric acid. Calcium was added in the form of  
154 CaCl<sub>2</sub>, to the whey protein solutions (1%, w/v, protein) at concentrations of 0.00, 0.25, 0.50,  
155 0.75, 1.00, 2.00, 3.00, 4.00 and 5.00 mM. Unless otherwise stated, the pH of all calcium-  
156 fortified solutions was measured and re-adjusted to pH 6.80, if required. The reagents and

157 standards used in this study were of analytical grade and purchased from Sigma Aldrich  
158 (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland), unless otherwise stated.

### 159 *2.3 Measurement of ionic calcium and titration with calcium chloride*

160 The ionic calcium concentration of the whey protein solutions (1%, w/v, protein, at  
161 pH 6.80) was measured using a calcium ion-selective polymer membrane electrode  
162 (Metrohm, Herisau, Switzerland) at 25°C. The ion-selective calcium probe was calibrated  
163 with standard calcium solutions at 0.00, 2.00, 4.00, 6.00, 8.00 and 10.0 mM at 25°C, by  
164 diluting a 1 M standard solution of CaCl<sub>2</sub> in ultra-pure water. The change in pH of the whey  
165 protein solutions (50 mL of 1%, w/v, protein, pH 6.80) on controlled addition (0.1 mL/min)  
166 of a CaCl<sub>2</sub> solution (0.5 M) was monitored using an automated Metrohm AG 907 Titrando pH  
167 titration system (Metrohm, Herisau, Switzerland) equipped with a combined pH and  
168 temperature probe. Calibration of the pH probe was carried out using three standard buffer  
169 solutions with pH of 4.00, 7.00 and 9.00.

### 171 *2.4 Measurement of particle size distribution and zeta potential*

172 The particle size distribution of the whey protein solutions (1%, w/v, protein, pH  
173 6.80) with added CaCl<sub>2</sub> was measured by dynamic light scattering (DLS) using a Zetasizer  
174 Nano-ZS (Malvern Instruments, Malvern, UK). For analysis, each solution was diluted 1:100  
175 in the respective whey protein-free calcium solution. A refractive index value of 1.45 was  
176 used for protein and the dispersant refractive index varied in response to differences in CaCl<sub>2</sub>  
177 concentration of the dispersant, ranging from 1.330 for 0.00 mM CaCl<sub>2</sub> (i.e., ultrapure water)  
178 to 1.332 for 5.00 mM CaCl<sub>2</sub>, with the refractive index calculated using the Mie theory. The  
179 zeta ( $\zeta$ )-potential was measured at 25°C for 120 s in automatic voltage mode, and  $\zeta$ -potential  
180 values were calculated using the Smoluchowski model.

181

182 *2.5 Isothermal titration calorimetry analysis of calcium-protein interactions*

183 The thermodynamic properties of interactions between whey proteins in solution (1%,  
184 w/v, protein, pH 6.80) and added  $\text{CaCl}_2$  were determined using isothermal titration  
185 calorimetry (ITC) with a MicroCal PEAQ-ITC instrument (Malvern Instruments, Malvern,  
186 UK). Whey protein solutions were titrated with 5.00 mM  $\text{CaCl}_2$ , at 25°C with stirring at 750  
187 rpm. The reference cell was filled with ultra-pure water of the same volume (250  $\mu\text{L}$ ) as the  
188 sample cell. The titrant was injected step-wise, in increments of 0.10  $\mu\text{L}$ , into the whey  
189 protein solution with a 150 s delay between successive injections and a total of 25 injections.  
190 The principle of the method is that the heat released or absorbed as a result of biomolecular  
191 binding is measured at constant temperature. The power applied to the reference cell was set  
192 at 10  $\mu\text{cal/s}$ , in line with previous studies (Canabady-Rochelle, Sanchez, Mellema, & Banon,  
193 2009). The model used was “one binding site” to establish the stoichiometry (N), binding  
194 constant ( $K_f$ ), Gibbs free energy ( $\Delta G$ ), enthalpy ( $\Delta H$ ) and entropy ( $-T\Delta S$ ). Titration profiles  
195 of the different whey protein solutions were expressed as differential power (i.e., difference  
196 in power between the reference and sample cells) as a function of time.

197

198 *2.6 Distribution of calcium between protein-bound and free forms*

199 The total calcium content of the whey protein solutions (1%, w/v, protein, pH 6.80)  
200 was determined using flame atomic absorption spectroscopy (AAS) (SpectrAA, 55B, AAS,  
201 Varian) fitted with a calcium hollow cathode lamp (Activion, Halstead, Essex, England) in  
202 accordance with the International Dairy Federation Standard 119:2007 (IDF, 2007). The  
203 instrument was calibrated using standard solutions (0.00, 2.00, 4.00, 6.00, 8.00, 10.0 mg/L of  
204 calcium) prepared from a calcium reference solution (1000 mg/L) with 2% addition level of a  
205 10% lanthanum chloride solution.  $\text{CaCl}_2$  was added (2 mM) to the protein solutions and  
206 allowed to equilibrate for 20 min at 20°C before the samples were centrifuged at 5550 rpm

207 for 25 min at 20°C in Amicon® centrifugal filter tubes (Merck Millipore, Carrigtwohill, Co.  
208 Cork, Ireland) with molecular weight cut-off of 10 kDa. Samples for AAS analysis had 24%  
209 trichloroacetic acid added in a ratio of 1:1 and allowed settle for 25 min before filtration  
210 through No. 413 filter paper (VWR International, France). The samples analysed for calcium  
211 content using AAS were the initial calcium-fortified (i.e., 2.00 mM added CaCl<sub>2</sub>) protein  
212 solutions and their respective supernatants.

213

#### 214 2.7 Accelerated colloidal stability analysis

215 The colloidal stability of the whey protein solutions (1%, w/v, protein, pH 6.80), with  
216 0.00 and 5.00 mM added CaCl<sub>2</sub>, was assessed using analytical centrifugation (LUMiSizer®,  
217 L.U.M. GmbH, Berlin, Germany). A three step method was used, consisting of 200 rpm from  
218 0 to 10 min, 1000 rpm from 10 to 20 min and 4000 rpm from 20 to 80 min. Results were  
219 expressed as integral transmission of the near infrared (NIR) light as a function of  
220 centrifugation time.

221

#### 222 2.8 Statistical data analysis

223 All samples were prepared three times independently, and all analyses were  
224 performed in triplicate for each independent experiment. The data generated was subjected to  
225 one-way analysis of variance (ANOVA) using R i386 version 3.3.1 (R foundation for  
226 statistical computing, Vienna, Austria). A Tukey's paired-comparison *post-hoc* test was used  
227 to determine statistically significant differences ( $p < 0.05$ ) between mean values for different  
228 samples, at the 95% confidence level. Results are expressed as mean value  $\pm$  standard  
229 deviation, and statistically significant differences are identified in tables using superscript  
230 letters, unless otherwise stated.

231

### 232 3. Results and discussion

#### 233 3.1 Ionic calcium concentration as a function of added calcium chloride

234 Binding of ionic calcium ( $\text{Ca}^{2+}$ ) by the different protein systems was monitored by  
235 measuring changes in ionic calcium concentration ( $[\text{Ca}^{2+}]$ ) as a function of added  $\text{CaCl}_2$  (Fig.  
236 1). The initial  $[\text{Ca}^{2+}]$  (i.e., innate  $[\text{Ca}^{2+}]$ ) for LAC-M and WPC was 0.58 and 1.96 mM,  
237 respectively, and were significantly higher ( $p < 0.05$ ) than for the other samples. Differences  
238 in innate  $[\text{Ca}^{2+}]$  for LAC ingredients were expected, as it has been previously reported that  
239 the use of different  $\alpha$ -lac enrichment technologies give rise to differences in  $[\text{Ca}^{2+}]$  between  
240 such ingredient (Barone, Moloney, O'Regan, Kelly & O'Mahony, 2020). An increase in  
241  $[\text{Ca}^{2+}]$  was measured with increasing level of  $\text{CaCl}_2$  addition for all the ingredients; the  
242 relationship between  $[\text{Ca}^{2+}]$  and added  $\text{CaCl}_2$  concentration was close to linear for samples  
243 WPC, WPI, LAC-M and LAC-IE (Fig. 1), in contrast, LAC-P and LAC-P-D both displayed  
244 considerably less linear (more concave) increases in  $[\text{Ca}^{2+}]$  as a function of added  $\text{CaCl}_2$ . This  
245 deviation from linearity was most evident in the concentration range 0.00-2.00 mM  $\text{CaCl}_2$   
246 and these results suggest that the proteins in LAC-P and LAC-P-D had higher  $\text{Ca}^{2+}$ -binding  
247 ability than those in WPI, WPC, LAC-M and LAC-IE.

248 During enrichment of  $\alpha$ -lac from whey using selective protein precipitation (i.e.,  
249 LAC-P), the  $\alpha$ -lac protein is extensively depleted in calcium (i.e., apo- $\alpha$ -lac) to achieve high  
250 heat-lability of  $\alpha$ -lac. This facilitates aggregation, precipitation and selective enrichment of  
251  $\alpha$ -lac from the other whey proteins (Kamau, Cheison, Chen, Liu, & Lu, 2010). In contrast,  
252 the production of LAC-M and LAC-IE does not involve the same extensive depletion of  
253 calcium, therefore, the  $\alpha$ -lac in these protein ingredients is present mainly in the holo- $\alpha$ -lac  
254 form, and consequently, the LAC-M and LAC-IE ingredients displayed similar interactions  
255 with calcium as WPI and WPC samples.

256 The LAC-P ingredient, in both original (LAC-P) and defatted (LAC-P-D) versions  
257 displayed very similar relationships between added  $\text{CaCl}_2$  and  $[\text{Ca}^{2+}]$ . This may contrast with  
258 previous studies demonstrating interactions between  $\text{Ca}^{2+}$  and  $\alpha$ -lac in the presence of  
259 phospholipid (PL) material, leading to PL-calcium- $\alpha$ -lac complex formation (Bo &  
260 Pawliszyn, 2006).

261

### 262 *3.2 Titration of protein solutions with calcium chloride*

263 The pH of the protein solutions (adjusted to an initial pH of 6.80) was measured as a  
264 function of  $\text{CaCl}_2$  addition level. The addition of  $\text{CaCl}_2$ , in the range 0.00-5.00 mM,  
265 decreased the pH of all protein solutions. A considerable difference in pH, expressed as  $\Delta\text{pH}$   
266 (i.e.,  $\Delta\text{pH} = \text{pH}_{@0\text{mM}} - \text{pH}_{@5\text{mM}}$ ), was measured for LAC-P and LAC-P-D, with values of 0.64  
267 and 0.61, respectively, followed by WPI (0.47). Values for  $\Delta\text{pH}$  of 0.36 and 0.29 were  
268 measured for LAC-IE and LAC-M, respectively; whereas WPC had a  $\Delta\text{pH}$  of 0.13, the  
269 lowest measured  $\Delta\text{pH}$  value. It is expected that the addition of soluble calcium salts (e.g.,  
270  $\text{CaCl}_2$ ) to protein solutions decreases the pH due to the release of hydrogen ions as a  
271 consequence of interactions between proteins and ions (Kharlamova, Nicolai & Chassenieux,  
272 2018) and also due to formation of calcium phosphate, a process which results release of  
273 hydrogen ions (Lewis, 2011). Kharlamova, Nicolai & Chassenieux (2018) reported that the  
274 decrease in pH of WPI solutions on addition of  $\text{CaCl}_2$  was due to the release of hydrogen ions  
275 by the proteins as a consequence of the binding of  $\text{Ca}^{2+}$  to specific sites of the protein  
276 molecules. This was also observed in the present work, especially for LAC-P, which showed  
277 the greatest  $\Delta\text{pH}$  among all samples.

278

### 279 *3.3 Particle size distribution*

280 The data for selected particle size distribution (PSD) parameters of the whey protein  
281 solutions as a function of  $\text{CaCl}_2$  addition level are reported in Table 1. The measured values  
282 for PSD parameters of the WPI with no added  $\text{CaCl}_2$  (e.g., VMD of 280 nm) were similar to  
283 those reported by Loveday, Ye, Anema, & Singh (2013) for a similar protein system; the  
284 VMD values for the LAC-M and LAC-P samples with no added  $\text{CaCl}_2$ , ranged from 264 to  
285 379 nm, while the VMD for the LAC-IE sample was 3.24 nm. Within samples, the VMD  
286 remained largely unchanged in the range 0.00 to 2.00 mM added  $\text{CaCl}_2$ , with values ranging  
287 from 4.25 to 360 nm, with LAC-IE and LAC-P-D displaying the lowest and highest VMD,  
288 respectively. At  $\text{CaCl}_2$  addition levels greater than 3.00 mM, the VMD increased markedly  
289 for LAC-P-D, followed by LAC-P and WPI, with values of 916, 584 and 472 nm,  
290 respectively, at 4 mM added  $\text{CaCl}_2$ . A bimodal PSD (i.e., where peaks 1 and 2 correspond to  
291 small and large size material, respectively) was observed for all ingredients except LAC-IE,  
292 which had a monomodal PSD. On increasing  $\text{CaCl}_2$  addition level from 0.00 to 5.00 mM, the  
293 greatest increases in volume diameter for individual particle size distribution peaks were  
294 measured for LAC-P-D, WPI and LAC-P with increases of 48.7, 131 and 184% for peak 1  
295 and 147, 110 and 84.1% for peak 2, respectively. The WPC, LAC-M and LAC-IE samples  
296 displayed minor differences in volume diameter for individual particle size distribution peaks  
297 on increasing addition level of  $\text{CaCl}_2$ . The polydispersity index (PDI) values ranged from 0.23  
298 to 0.80 for all samples, with the width of the PSD generally increasing with increasing  $\text{CaCl}_2$   
299 addition level, and the samples displaying the greatest changes in PDI were WPI, LAC-P,  
300 LAC-P-D and LAC-IE.

301 It has been previously reported that increasing  $\text{Ca}^{2+}$  concentration can increase  
302 particle size and influence the functional properties of whey proteins (Clare, Lillard, Ramsey,  
303 Amato, & Daubert, 2007) as it can mediate cross-linking of protein molecules (Bryant &  
304 McClements, 1998). The selective removal of fat and PL components from one of the three

305 LAC ingredients (i.e., LAC-P-D) resulted in larger VMD at CaCl<sub>2</sub> addition levels greater than  
306 2.00 mM, in comparison with the original ingredient (LAC-P). The PL components of LAC-P  
307 restricted increases in VMD, compared with LAC-P-D, when CaCl<sub>2</sub> was added at the same  
308 level. This stabilising effect of PL on the particle size in whey protein solutions can be  
309 attributed to interactions between whey proteins and PL components. The formation of PL-  
310 whey protein complexes has been reported to be mainly driven by electrostatic and  
311 hydrophobic interactions, and this complex can potentially decrease calcium-bridging  
312 between whey proteins (Alzagtat & Alli, 2002; Corredig & Dalgleish, 1996) thereby resulting  
313 in higher protein stability of calcium-fortified whey-based solutions.

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331 **Table 1:** Particle size distribution of protein solutions (1%, w/v, protein, pH 6.80) prepared from whey  
 332 protein isolate (WPI), whey protein concentrate (WPC) and whey protein concentrates enriched in  $\alpha$ -  
 333 lactalbumin prepared using membrane filtration (LAC-M), selective protein precipitation (LAC-P),  
 334 followed by defatting (LAC-P-D), and ion-exchange (LAC-IE) as a function of calcium chloride  
 335 addition level.

Sample	Calcium chloride concentration (mM)	Polydispersity index (Pdl)	Volume mean diameter (nm)	Peak 1		Peak 2	
				Volume Diameter (nm)	Percent of Total Area (%)	Volume Diameter (nm)	Percent of Total Area (%)
				(nm)	(%)	(nm)	(%)
WPI	0.00	0.43 ± 0.07 <sup>a</sup>	280 ± 57.4 <sup>b</sup>	65.1 ± 9.29 <sup>a</sup>	49.1 ± 8.73 <sup>b</sup>	358 ± 29.6 <sup>bc</sup>	50.8 ± 8.73 <sup>a</sup>
	0.25	0.51 ± 0.01 <sup>b</sup>	216 ± 37.4 <sup>b</sup>	83.1 ± 0.74 <sup>ab</sup>	49.7 ± 2.75 <sup>c</sup>	365 ± 32.3 <sup>b</sup>	50.2 ± 2.75 <sup>a</sup>
	0.50	0.59 ± 0.01 <sup>c</sup>	279 ± 67.6 <sup>b</sup>	90.5 ± 9.84 <sup>b</sup>	39.3 ± 3.90 <sup>a</sup>	349 ± 42.3 <sup>cd</sup>	60.7 ± 3.90 <sup>a</sup>
	0.75	0.49 ± 0.04 <sup>b</sup>	273 ± 20.5 <sup>b</sup>	61.3 ± 3.98 <sup>a</sup>	46.1 ± 6.14 <sup>c</sup>	373 ± 22.8 <sup>d</sup>	53.9 ± 7.14 <sup>a</sup>
	1.00	0.47 ± 0.01 <sup>c</sup>	290 ± 19.5 <sup>b</sup>	77.3 ± 9.05 <sup>ab</sup>	40.3 ± 8.72 <sup>a</sup>	387 ± 51.7 <sup>bc</sup>	59.7 ± 10.7 <sup>a</sup>
	2.00	0.47 ± 0.01 <sup>b</sup>	259 ± 13.0 <sup>b</sup>	97.8 ± 12.7 <sup>a</sup>	43.9 ± 4.34 <sup>b</sup>	382 ± 19.4 <sup>bc</sup>	56.1 ± 4.38 <sup>a</sup>
	3.00	0.54 ± 0.01 <sup>d</sup>	308 ± 32.5 <sup>bc</sup>	133 ± 11.9 <sup>a</sup>	38.7 ± 5.25 <sup>bc</sup>	454 ± 15.2 <sup>d</sup>	61.3 ± 2.38 <sup>a</sup>
	4.00	0.51 ± 0.04 <sup>b</sup>	472 ± 72.1 <sup>cd</sup>	93.5 ± 9.33 <sup>a</sup>	38.8 ± 0.14 <sup>bc</sup>	530 ± 73.6 <sup>c</sup>	61.2 ± 7.07 <sup>a</sup>
5.00	0.76 ± 0.22 <sup>bc</sup>	638 ± 24.0 <sup>c</sup>	151 ± 15.6 <sup>b</sup>	19.4 ± 0.12 <sup>b</sup>	755 ± 23.9 <sup>c</sup>	80.6 ± 0.14 <sup>c</sup>	
WPC	0.00	0.24 ± 0.01 <sup>a</sup>	362 ± 17.0 <sup>b</sup>	50.5 ± 5.17 <sup>a</sup>	15.9 ± 2.57 <sup>a</sup>	409 ± 8.40 <sup>cd</sup>	84.1 ± 2.57 <sup>cd</sup>
	0.25	0.24 ± 0.01 <sup>a</sup>	377 ± 58.9 <sup>c</sup>	58.1 ± 5.36 <sup>a</sup>	12.8 ± 2.92 <sup>a</sup>	374 ± 65.5 <sup>b</sup>	87.2 ± 9.82 <sup>c</sup>
	0.50	0.32 ± 0.01 <sup>c</sup>	345 ± 55.2 <sup>c</sup>	96.1 ± 5.52 <sup>b</sup>	26.5 ± 0.28 <sup>a</sup>	395 ± 4.80 <sup>d</sup>	73.5 ± 0.28 <sup>a</sup>
	0.75	0.25 ± 0.01 <sup>a</sup>	255 ± 17.6 <sup>b</sup>	71.6 ± 2.05 <sup>ab</sup>	24.6 ± 1.62 <sup>a</sup>	304 ± 2.33 <sup>c</sup>	75.4 ± 1.62 <sup>c</sup>
	1.00	0.37 ± 0.02 <sup>bc</sup>	315 ± 68.2 <sup>b</sup>	107 ± 11.2 <sup>bc</sup>	25.6 ± 1.25 <sup>a</sup>	423 ± 48.3 <sup>c</sup>	74.4 ± 1.25 <sup>a</sup>
	2.00	0.23 ± 0.01 <sup>a</sup>	330 ± 72.8 <sup>bc</sup>	90.1 ± 8.57 <sup>a</sup>	30.2 ± 2.58 <sup>ab</sup>	378 ± 24.3 <sup>bc</sup>	69.8 ± 3.21 <sup>b</sup>
	3.00	0.24 ± 0.01 <sup>a</sup>	369 ± 42.2 <sup>c</sup>	104 ± 8.13 <sup>a</sup>	29.9 ± 1.40 <sup>b</sup>	356 ± 26.9 <sup>cd</sup>	70.1 ± 1.40 <sup>b</sup>
	4.00	0.27 ± 0.05 <sup>a</sup>	253 ± 18.7 <sup>bc</sup>	96.0 ± 8.52 <sup>a</sup>	26.1 ± 2.85 <sup>b</sup>	299 ± 15.5 <sup>b</sup>	73.9 ± 2.85 <sup>ab</sup>
5.00	0.25 ± 0.01 <sup>a</sup>	309 ± 43.3 <sup>b</sup>	100 ± 1.92 <sup>a</sup>	28.2 ± 1.82 <sup>c</sup>	356 ± 32.2 <sup>b</sup>	71.8 ± 2.82 <sup>b</sup>	
LAC-M	0.00	0.38 ± 0.19 <sup>a</sup>	264 ± 15.7 <sup>b</sup>	67.0 ± 7.35 <sup>ab</sup>	26.7 ± 1.34 <sup>a</sup>	287 ± 25.1 <sup>b</sup>	73.2 ± 1.34 <sup>bc</sup>
	0.25	0.29 ± 0.06 <sup>a</sup>	250 ± 26.2 <sup>b</sup>	85.9 ± 5.06 <sup>ab</sup>	34.5 ± 5.58 <sup>bc</sup>	307 ± 27.8 <sup>b</sup>	65.1 ± 5.58 <sup>ab</sup>
	0.50	0.25 ± 0.01 <sup>ab</sup>	241 ± 17.6 <sup>b</sup>	87.5 ± 2.53 <sup>b</sup>	39.7 ± 8.52 <sup>a</sup>	300 ± 9.89 <sup>bc</sup>	60.3 ± 11.5 <sup>a</sup>
	0.75	0.24 ± 0.01 <sup>a</sup>	194 ± 11.5 <sup>ab</sup>	69.8 ± 10.8 <sup>ab</sup>	31.1 ± 0.70 <sup>ab</sup>	252 ± 12.7 <sup>b</sup>	68.9 ± 0.72 <sup>bc</sup>
	1.00	0.27 ± 0.01 <sup>ab</sup>	258 ± 27.0 <sup>b</sup>	119 ± 13.1 <sup>c</sup>	38.9 ± 8.90 <sup>a</sup>	378 ± 13.2 <sup>bc</sup>	61.1 ± 8.82 <sup>a</sup>
	2.00	0.24 ± 0.01 <sup>a</sup>	225 ± 35.3 <sup>b</sup>	73.1 ± 4.80 <sup>a</sup>	43.2 ± 6.57 <sup>b</sup>	294 ± 25.8 <sup>b</sup>	56.8 ± 6.57 <sup>a</sup>
	3.00	0.24 ± 0.01 <sup>a</sup>	260 ± 14.6 <sup>b</sup>	76.4 ± 2.75 <sup>a</sup>	39.5 ± 2.12 <sup>c</sup>	237 ± 18.0 <sup>b</sup>	60.5 ± 2.12 <sup>a</sup>
	4.00	0.24 ± 0.01 <sup>a</sup>	201 ± 25.5 <sup>ab</sup>	74.9 ± 3.85 <sup>a</sup>	37.8 ± 4.87 <sup>c</sup>	225 ± 21.5 <sup>b</sup>	62.2 ± 4.01 <sup>a</sup>
5.00	0.26 ± 0.01 <sup>a</sup>	204 ± 61.5 <sup>b</sup>	105 ± 15.6 <sup>a</sup>	42.6 ± 0.65 <sup>d</sup>	357 ± 13.2 <sup>b</sup>	57.4 ± 0.49 <sup>a</sup>	
LAC-P	0.00	0.26 ± 0.01 <sup>a</sup>	288 ± 95.6 <sup>b</sup>	45.0 ± 8.87 <sup>a</sup>	32.2 ± 10.8 <sup>ab</sup>	334 ± 58.2 <sup>bc</sup>	66.3 ± 8.76 <sup>ab</sup>
	0.25	0.25 ± 0.01 <sup>a</sup>	281 ± 20.9 <sup>bc</sup>	98.0 ± 10.1 <sup>b</sup>	38.5 ± 1.60 <sup>bc</sup>	357 ± 29.8 <sup>b</sup>	61.4 ± 1.60 <sup>ab</sup>
	0.50	0.24 ± 0.01 <sup>a</sup>	188 ± 31.5 <sup>b</sup>	49.4 ± 0.80 <sup>a</sup>	38.3 ± 0.81 <sup>a</sup>	275 ± 24.6 <sup>b</sup>	61.7 ± 0.81 <sup>a</sup>
	0.75	0.24 ± 0.01 <sup>a</sup>	234 ± 72.1 <sup>ab</sup>	57.4 ± 5.37 <sup>a</sup>	39.2 ± 3.93 <sup>bc</sup>	258 ± 2.90 <sup>b</sup>	60.8 ± 3.95 <sup>ab</sup>
	1.00	0.24 ± 0.01 <sup>a</sup>	201 ± 21.5 <sup>b</sup>	91.7 ± 4.51 <sup>bc</sup>	36.5 ± 3.01 <sup>a</sup>	318 ± 16.1 <sup>b</sup>	63.5 ± 2.97 <sup>a</sup>
	2.00	0.25 ± 0.01 <sup>a</sup>	236 ± 28.2 <sup>b</sup>	93.8 ± 5.10 <sup>a</sup>	30.1 ± 4.51 <sup>ab</sup>	326 ± 28.2 <sup>bc</sup>	69.9 ± 1.60 <sup>b</sup>
	3.00	0.26 ± 0.01 <sup>a</sup>	249 ± 43.1 <sup>b</sup>	55.1 ± 3.05 <sup>a</sup>	40.0 ± 3.65 <sup>c</sup>	326 ± 31.1 <sup>c</sup>	60.0 ± 2.81 <sup>a</sup>
	4.00	0.45 ± 0.02 <sup>ab</sup>	584 ± 18.8 <sup>d</sup>	165 ± 6.22 <sup>b</sup>	20.1 ± 0.71 <sup>a</sup>	526 ± 35.7 <sup>c</sup>	79.9 ± 9.97 <sup>bc</sup>
5.00	0.50 ± 0.04 <sup>ab</sup>	638 ± 22.6 <sup>c</sup>	128 ± 7.25 <sup>ab</sup>	19.5 ± 2.55 <sup>ab</sup>	615 ± 40.3 <sup>c</sup>	80.5 ± 2.55 <sup>c</sup>	
LAC-P-D	0.00	0.27 ± 0.01 <sup>a</sup>	379 ± 21.5 <sup>b</sup>	91.4 ± 6.20 <sup>b</sup>	17.6 ± 3.30 <sup>a</sup>	439 ± 13.1 <sup>d</sup>	82.3 ± 3.30 <sup>bc</sup>
	0.25	0.27 ± 0.01 <sup>a</sup>	317 ± 52.5 <sup>bc</sup>	61.9 ± 8.74 <sup>a</sup>	30.3 ± 5.62 <sup>b</sup>	363 ± 32.2 <sup>b</sup>	69.7 ± 5.62 <sup>b</sup>
	0.50	0.28 ± 0.01 <sup>b</sup>	280 ± 31.3 <sup>b</sup>	83.3 ± 5.48 <sup>b</sup>	39.2 ± 6.07 <sup>a</sup>	393 ± 4.82 <sup>d</sup>	67.1 ± 3.02 <sup>a</sup>
	0.75	0.27 ± 0.01 <sup>a</sup>	276 ± 45.0 <sup>b</sup>	90.1 ± 3.39 <sup>b</sup>	27.6 ± 1.83 <sup>ab</sup>	389 ± 3.04 <sup>d</sup>	72.4 ± 1.83 <sup>c</sup>
	1.00	0.27 ± 0.01 <sup>ab</sup>	273 ± 24.1 <sup>b</sup>	66.3 ± 9.38 <sup>a</sup>	35.0 ± 1.64 <sup>a</sup>	363 ± 0.62 <sup>bc</sup>	65.0 ± 1.64 <sup>a</sup>
	2.00	0.27 ± 0.01 <sup>a</sup>	360 ± 16.7 <sup>c</sup>	79.9 ± 8.84 <sup>a</sup>	22.0 ± 5.65 <sup>a</sup>	400 ± 7.63 <sup>c</sup>	78.0 ± 5.65 <sup>b</sup>
	3.00	0.35 ± 0.04 <sup>b</sup>	760 ± 43.8 <sup>d</sup>	63.4 ± 5.48 <sup>a</sup>	9.40 ± 0.14 <sup>a</sup>	798 ± 11.6 <sup>c</sup>	90.6 ± 0.14 <sup>c</sup>
	4.00	0.48 ± 0.01 <sup>b</sup>	916 ± 84.2 <sup>c</sup>	151 ± 0.28 <sup>b</sup>	6.80 ± 0.15 <sup>a</sup>	929 ± 73.6 <sup>d</sup>	93.2 ± 0.14 <sup>cd</sup>
5.00	0.50 ± 0.04 <sup>bc</sup>	985 ± 23.2 <sup>d</sup>	136 ± 8.55 <sup>ab</sup>	4.40 ± 0.35 <sup>a</sup>	1085 ± 154 <sup>d</sup>	95.6 ± 0.3 <sup>d</sup>	
LAC-IE	0.00	0.68 ± 0.02 <sup>b</sup>	3.24 ± 0.89 <sup>a</sup>	ND	ND	3.25 ± 0.35 <sup>a</sup>	100 ± 0.01 <sup>d</sup>
	0.25	0.66 ± 0.01 <sup>c</sup>	3.61 ± 0.38 <sup>a</sup>	ND	ND	3.99 ± 0.25 <sup>a</sup>	100 ± 0.01 <sup>c</sup>
	0.50	0.43 ± 0.01 <sup>d</sup>	4.41 ± 0.17 <sup>a</sup>	ND	ND	4.60 ± 0.01 <sup>a</sup>	100 ± 0.01 <sup>b</sup>
	0.75	0.45 ± 0.04 <sup>b</sup>	3.87 ± 0.74 <sup>a</sup>	ND	ND	4.17 ± 0.61 <sup>a</sup>	100 ± 0.01 <sup>d</sup>
	1.00	0.42 ± 0.05 <sup>c</sup>	4.49 ± 0.46 <sup>a</sup>	ND	ND	4.39 ± 0.43 <sup>a</sup>	100 ± 0.01 <sup>b</sup>
	2.00	0.43 ± 0.05 <sup>b</sup>	4.25 ± 0.06 <sup>a</sup>	ND	ND	4.16 ± 0.28 <sup>a</sup>	100 ± 0.01 <sup>c</sup>
	3.00	0.45 ± 0.01 <sup>c</sup>	4.13 ± 0.89 <sup>a</sup>	ND	ND	4.71 ± 0.20 <sup>a</sup>	100 ± 0.01 <sup>d</sup>
	4.00	0.79 ± 0.08 <sup>c</sup>	4.13 ± 0.97 <sup>a</sup>	ND	ND	4.28 ± 0.24 <sup>a</sup>	100 ± 0.01 <sup>d</sup>
5.00	0.80 ± 0.04 <sup>c</sup>	3.87 ± 0.67 <sup>a</sup>	ND	ND	4.17 ± 0.18 <sup>a</sup>	100 ± 0.01 <sup>d</sup>	

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337 Values followed by different superscript letters in the same column are significantly different ( $p < 0.05$ )

338 \*ND = not detected

### 339 3.4 Zeta potential

340 The zeta ( $\zeta$ )-potential of whey protein solutions as a function of  $\text{CaCl}_2$  addition level  
341 is shown in Fig. 2. Prior to addition of  $\text{CaCl}_2$ , all protein solutions displayed a net negative  $\zeta$ -  
342 potential. Initial  $\zeta$ -potential of WPI (-34.0 mV) was in line with previous literature (Klein,  
343 Aserin, Ishai, & Garti, 2010). The most negative initial  $\zeta$ -potential for samples with no added  
344  $\text{CaCl}_2$  was measured for LAC-P (-40.9 mV), while the least negative  $\zeta$ -potential was  
345 measured for LAC-IE (-17.0 mV). This  $\zeta$ -potential for LAC-IE was expected for an ion-  
346 exchange chromatography-produced ingredient, due to the relatively high sodium content  
347 (680 mg/100 g) arising from the use of this approach for enrichment  $\alpha$ -lac (Barone, Moloney,  
348 O'Regan, Kelly & O'Mahony, 2020). A plateau in  $\zeta$ -potential was evident at  $\text{CaCl}_2$  addition  
349 levels greater than 3.00 mM for all solutions, with LAC-P and LAC-P-D exhibiting the  
350 highest negative  $\zeta$ -potential, with values of -8.81 and -8.08 mV, respectively.

351 The negative  $\zeta$ -potential displayed by all samples at pH 6.80 was expected as, at this  
352 pH, the amino groups of proteins are uncharged ( $-\text{NH}_2$ ), whereas the carboxyl groups of  
353 proteins are negatively charged ( $-\text{COO}^-$ ); therefore, addition of calcium in the form of  $\text{CaCl}_2$   
354 is expected to, at least partially, shield the carboxyl groups, thereby lowering the negative  $\zeta$ -  
355 potential (Kulmyrzaev, Chanamai, & McClements, 2000). On increasing  $\text{CaCl}_2$  addition, the  
356 greater measured decreases in  $\zeta$ -potential for LAC-P and LAC-P-D than for the benchmark  
357 samples WPI and WPC is in line with PSD analysis, as the VMD increased considerably in  
358 the LAC-P sample, which is indicative of extensive calcium-mediated protein aggregation.  
359 This effect may also be due to transition of the  $\alpha$ -lac protein from apo- (i.e., calcium-  
360 depleted) to holo- (i.e., calcium-bound) state (Wijesinha-Bettoni, Dobson, & Redfield, 2001).

361

### 362 3.5 Thermodynamic characterisation of calcium-protein interactions

363 Isothermal titration calorimetry (ITC) was used in this study to better understand and  
364 quantify the thermodynamic properties of the calcium-protein interactions. ITC can be used  
365 to determine the thermodynamic properties of such interactions by measuring the heat flow  
366 produced when a ligand (i.e.,  $\text{Ca}^{2+}$  from  $\text{CaCl}_2$ ) is bound to a specific site on the protein at  
367 constant temperature. The titration thermographs and the thermodynamic constants obtained  
368 (i.e., Gibbs free energy, enthalpy, entropy, affinity constant and stoichiometry) are displayed  
369 in Fig. 3 and Table 2, respectively. The addition of  $\text{CaCl}_2$  to the protein solutions resulted in  
370 negative values for Gibbs free energy ( $\Delta G$ ), ranging from -16.0 to -5.53 (kcal/mol),  
371 suggesting that the binding of  $\text{Ca}^{2+}$  to whey protein molecules can proceed spontaneously.

372 The binding of  $\text{Ca}^{2+}$  to protein molecules in WPI, WPC and LAC-M samples resulted  
373 in positive enthalpy ( $\Delta H$ ) and negative entropy ( $-\text{T}\Delta S$ ) with values of 70.1, 22.2, 80.6  
374 kcal/mol for  $\Delta H$  and -86.3, -27.7 and -86.0 kcal/mol for  $-\text{T}\Delta S$ , respectively. In contrast, the  
375 values for  $\Delta H$  determined for LAC-P, LAC-P-D and LAC-IE were significantly different ( $p <$   
376 0.05) from those of the other protein solutions, with values of -17.4, -28.3, -2.02 kcal/mol and  
377  $-\text{T}\Delta S$  values of 8.24, 19.1 and -4.69 kcal/mol, respectively. These results confirmed that the  
378 proteins in both versions of LAC-P (i.e., LAC P-O and LAC P-D) had high affinity for, and  
379 strongly bound  $\text{Ca}^{2+}$ . These interactions between  $\text{Ca}^{2+}$  and proteins in LAC-P were attributed  
380 to the apo-state of  $\alpha$ -lac, which has a strong ability to bind  $\text{Ca}^{2+}$  (Permyakov & Berliner,  
381 2000). This high affinity for  $\text{Ca}^{2+}$  by LAC-P in both versions was also confirmed by the  
382 significantly lower ( $p < 0.05$ ) affinity constant ( $K_d$ ) for  $\text{Ca}^{2+}$  compared to the other LAC  
383 samples, with values of  $1.63 \times 10^{-7}$  and  $2.10 \times 10^{-7}$  for LAC-P and LAC-P-D. Weaker binding  
384 affinity for  $\text{Ca}^{2+}$  was observed for the LAC-IE protein system, as evident from the titration  
385 thermographs (Fig. 4-f); endothermic peaks were recorded for the initial three injections,  
386 generating a stoichiometry value of 0.10, which is associated with the residual apo form of  $\alpha$ -  
387 lac in this sample.

388 The negative  $\Delta G$  and positive  $-\Delta S$  for both versions of the LAC-P protein system  
389 indicate that the binding of  $\text{Ca}^{2+}$  occurred spontaneously and was enthalpically driven  
390 (Ladbury & Chowdhry, 1996). In contrast, the thermodynamic energy involved for WPI,  
391 WPC and LAC-M was due to the dilution effect of the titrant in the protein solution cell  
392 (Canabady-Rochelle, Sanchez, Mellema, & Banon, 2009). Interestingly, the stoichiometry  
393 (N) values measured for LAC-P (0.71) and LAC-P-D (0.50) were similar to previous reports  
394 for pure bovine  $\alpha$ -lac in the apo form (N = 1) (Permyakov & Berliner, 2000). The removal of  
395 PL components from LAC-P (i.e., LAC-P-D) altered the calcium-binding properties as the  
396 stoichiometry values were significantly different ( $p < 0.05$ ) between the defatted (i.e., LAC-  
397 P-D) and original (i.e., LAC-P) versions. It has been previously reported that PL components  
398 can influence the calcium-binding properties of apo- $\alpha$ -lac (Barbana et al., 2006; Kim & Kim,  
399 1986), and the results of the current study (e.g., particle size distribution and zeta potential)  
400 are in agreement with this.

401

### 402 3.6 Calcium distribution analysis

403 The calcium content of the 1%, w/v, protein solutions with 2.00 mM  $\text{CaCl}_2$  was  
404 determined by atomic absorption spectroscopy (AAS), before and after filtration through 10-  
405 kDa MWCO filters (Table 2). The total calcium content of the protein solutions ranged from  
406 89.2 to 205 mg/L, with LAC-P-D and WPC having the lowest and highest ( $p < 0.05$ ) calcium  
407 contents, respectively. The same trends in calcium content were evident in the respective  
408 permeate fractions after filtration. Approximately two thirds of total calcium was bound by  
409 the proteins in LAC-M (65.6%), WPI (67.4%) and LAC-IE (58.6%), while WPC (43.9%) had  
410 the lowest proportion of calcium bound by protein. As expected from results presented earlier  
411 in this study, LAC-P and LAC-P-D displayed the greatest extent of calcium binding by the  
412 protein, with values of 81.8 and 69.4%, respectively. LAC-P-D had a significantly lower

- 413 (~10%) level of calcium bound by the protein than LAC-P, in agreement with data for  
414 thermodynamics of calcium-protein interactions from ITC analysis.

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415 **Table 2:** Gibbs free energy ( $\Delta G$ ), enthalpy ( $\Delta H$ ), entropy ( $-T\Delta S$ ), affinity constant ( $K_d$ ) and stoichiometry ( $N$ ) from isothermal titration calorimetry analysis  
 416 of calcium-protein interactions and calcium distribution analysis between the protein-bound and free calcium in the permeate fractions after filtration through  
 417 10 kDa MWCO ultrafiltration membranes of the protein solutions added with 2 mM  $\text{CaCl}_2$  prepared using whey protein concentrate (WPC), whey protein  
 418 concentrates enriched in  $\alpha$ -lactalbumin prepared using membrane filtration (LAC-M), selective protein precipitation (LAC-P), LAC-P followed by defatting  
 419 (LAC-P-D), and ion-exchange (LAC-IE).

Sample	$\Delta G$	$\Delta H$ (kcal/mol)	$-T\Delta S$	$K_d$ (-)	$N$ (-)	Calcium content of protein solution (mg/L)	Calcium content of permeate (mg/L)	Proportion of total calcium bound by protein (%)
WPI	$-16.0 \pm 0.55^a$	$70.1 \pm 0.01^c$	$-86.3 \pm 0.11^a$	$1.02 \times 10^{-4}^d$	$0.00 \pm 0.01^a$	$100 \pm 1.15^b$	$32.7 \pm 2.15^c$	67.4
WPC	$-5.53 \pm 0.01^d$	$22.2 \pm 0.25^d$	$-27.7 \pm 0.25^b$	$8.79 \times 10^{-5}^c$	$0.00 \pm 0.01^a$	$205 \pm 1.69^e$	$115 \pm 2.47^f$	43.9
LAC-M	$-6.28 \pm 0.01^c$	$80.6 \pm 1.21^f$	$-86.0 \pm 0.27^a$	$2.46 \times 10^{-5}^b$	$0.00 \pm 0.01^a$	$146 \pm 1.45^d$	$50.2 \pm 1.69^e$	65.6
LAC-P	$-9.30 \pm 0.05^b$	$-17.4 \pm 0.05^b$	$8.24 \pm 0.01^d$	$1.63 \times 10^{-7}^a$	$0.71 \pm 0.01^d$	$97.1 \pm 2.49^b$	$17.6 \pm 1.14^a$	81.8
LAC-P-D	$-9.19 \pm 0.02^b$	$-28.3 \pm 0.11^a$	$19.1 \pm 0.11^c$	$2.10 \times 10^{-7}^a$	$0.50 \pm 0.07^c$	$89.2 \pm 1.10^a$	$27.3 \pm 0.53^b$	69.4
LAC-IE	$-6.70 \pm 0.01^c$	$-2.02 \pm 0.02^c$	$-4.69 \pm 0.07^c$	$1.21 \times 10^{-4}^c$	$0.10 \pm 0.01^b$	$110 \pm 2.85^c$	$45.8 \pm 2.42^d$	58.6

420 Values followed by different superscript letters in the same column are significantly different ( $p < 0.05$ )

421 \*Calcium bound by protein expressed as:  $\frac{Ca_{solution} - Ca_{permeate}}{Ca_{solution}} * 100$

422 *3.7 Accelerated suspension stability*

423 Analytical centrifugation was used to evaluate the optical properties and suspension  
424 stability of the 1% protein solutions with 0.00 and 5.00 mM CaCl<sub>2</sub> added. Different initial  
425 (i.e., 0 min) optical properties of the ingredients were observed (Fig. 4), with WPC having the  
426 lowest transmission (62.0%), while LAC-IE had the highest transmission (86.5%). Addition  
427 of 5.00 mM CaCl<sub>2</sub> resulted in minimal changes in integral transmission of the samples,  
428 except for LAC-P and LAC-P-D in which significantly lower transmission (49.4 and 46.1%  
429 for LAC-P and LAC-P-D, respectively) was measured when compared to their counterparts  
430 with 0.00 mM CaCl<sub>2</sub> addition. Centrifugation resulted in slight clarification (i.e., higher  
431 integral transmission) for all ingredients, with greater clarification observed for LAC-P and  
432 LAC-P-D at 5.00 mM CaCl<sub>2</sub> (Fig. 5). This physical instability (i.e., clarification on  
433 centrifugation) is in agreement with the PSD analysis presented earlier, as at 5.00 mM added  
434 CaCl<sub>2</sub>, the VMD of LAC-P increased, which influenced the optical (i.e., lower transmission)  
435 and colloidal properties.

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449 **Conclusion**

450 The addition of calcium in the form of calcium chloride to  $\alpha$ -lac-enriched WPC solutions  
451 resulted in considerable changes to the physicochemical properties of the resultant solutions.  
452 The extent of these changes was dependent on the protein profile, physical state of  $\alpha$ -  
453 lactalbumin (e.g., calcium-bound or depleted) and concentrations of other macromolecules  
454 (e.g., phospholipid) in the  $\alpha$ -lac-enriched ingredients, which are in turn strongly influenced  
455 by the choice of technological approach used to enrich  $\alpha$ -lac in these ingredients. The  $\alpha$ -lac-  
456 enriched ingredients generally displayed the same or better calcium-binding and stabilising  
457 properties as regular WPC and WPI ingredients with unaltered protein profile. More  
458 specifically, phospholipids co-enriched with protein in the production of  $\alpha$ -lac-enriched  
459 ingredients contributed to the strongest calcium-binding properties of this ingredient. The  
460 results obtained in this study provide fundamental insights into the influence of fortification  
461 with soluble calcium salts on the physicochemical stability of next-generation WPC  
462 ingredients enriched in  $\alpha$ -lac. These findings are essential in supporting further development  
463 of such value-added ingredients and underpins the optimisation of calcium-enrichment  
464 strategies used in the formulation of nutritional whey-based products.

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615

616 **Figure 1:** Concentration of ionic calcium (mM) as a function of calcium chloride concentration (mM)  
 617 for 1% protein solutions at pH 6.80 prepared from whey protein isolate (WPI; —■—), whey protein  
 618 concentrate (WPC; —▲—), whey protein concentrate enriched in  $\alpha$ -lactalbumin prepared using

619 manufactured by membrane filtration (LAC-M; —□—), selective protein precipitation (LAC-P; —  
620 Δ—), LAC-P followed by defatting (LAC-P-D; —◇—) and ion-exchange (LAC-IE; —○—).

621

622 **Figure 2:** Zeta potential as a function of calcium chloride concentration (mM) for 1% protein solution  
623 at pH 6.80 prepared from whey protein isolate (WPI; —■—), whey protein concentrate (WPC; —  
624 ▲—), whey protein concentrate enriched in  $\alpha$ -lactalbumin prepared using manufactured by  
625 membrane filtration (LAC-M; —□—), selective protein precipitation (LAC-P; —Δ—), LAC-P  
626 followed by defatting (LAC-P-D; —◇—) and ion-exchange (LAC-IE; —○—).

627

628 **Figure 3:** Isothermal titration calorimetry thermographs of (a) whey protein isolate (WPI), (b) whey  
629 protein concentrate (WPC), (c) whey protein concentrate enriched in  $\alpha$ -lactalbumin prepared using  
630 membrane filtration (LAC-M), (d) selective protein precipitation (LAC-P), (e) LAC-P followed by  
631 defatting (LAC-P-D) and (f) ion-exchange (LAC-IE).

632

633 **Figure 4:** Representative accelerated physical stability profiles expressed as integral transmission of  
634 the NIR light at 0 mM  $\text{CaCl}_2$  (solid line) and 5 mM  $\text{CaCl}_2$  (dashed line) of 1% protein solutions at pH  
635 6.80 prepared from (a) whey protein isolate (WPI), (b) whey protein concentrate (WPC), (c) whey  
636 protein concentrate enriched in  $\alpha$ -lactalbumin prepared using membrane filtration (LAC-M), (d)  
637 selective protein precipitation (LAC-P), (e) LAC-P followed by defatting (LAC-P-D) and (f) ion-  
638 exchange (LAC-IE)

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641 **Credit Author Statement**

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643 **Influence of calcium fortification on physicochemical properties of whey protein**  
644 **concentrate solutions enriched in  $\alpha$ -lactalbumin**

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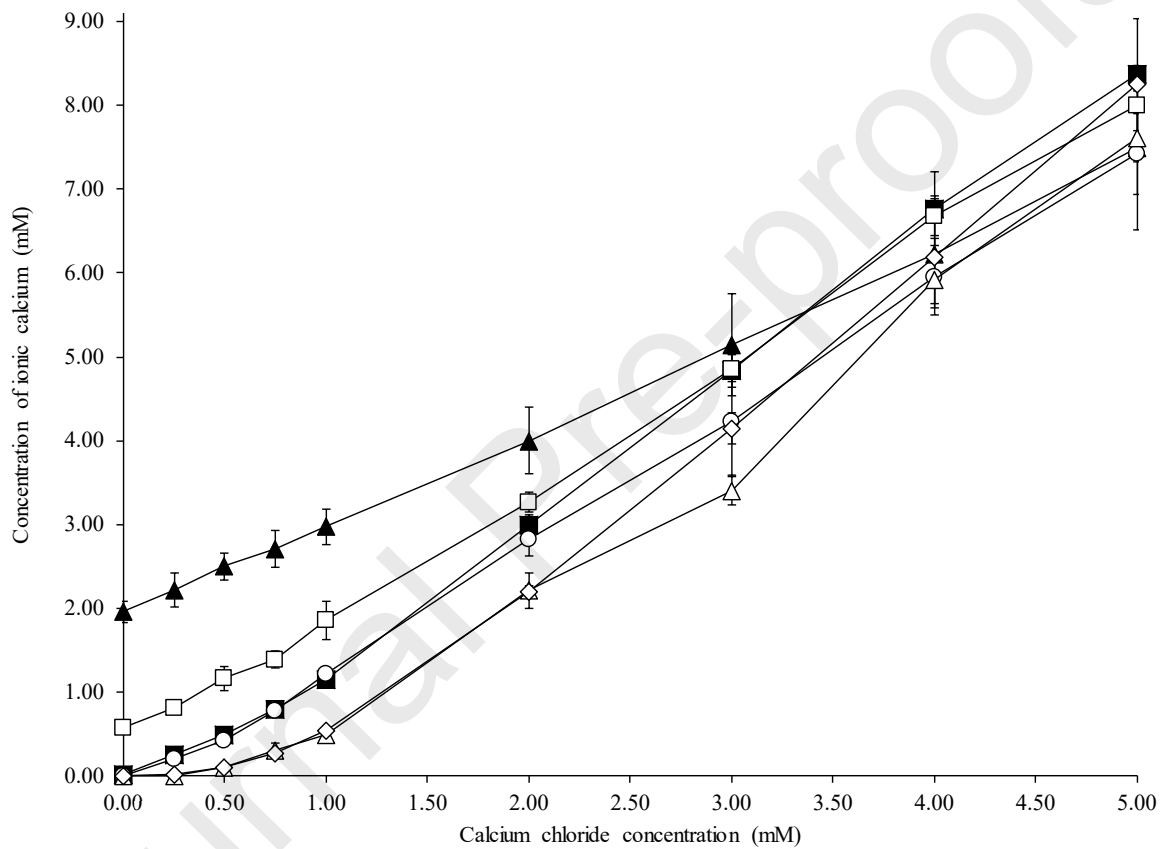
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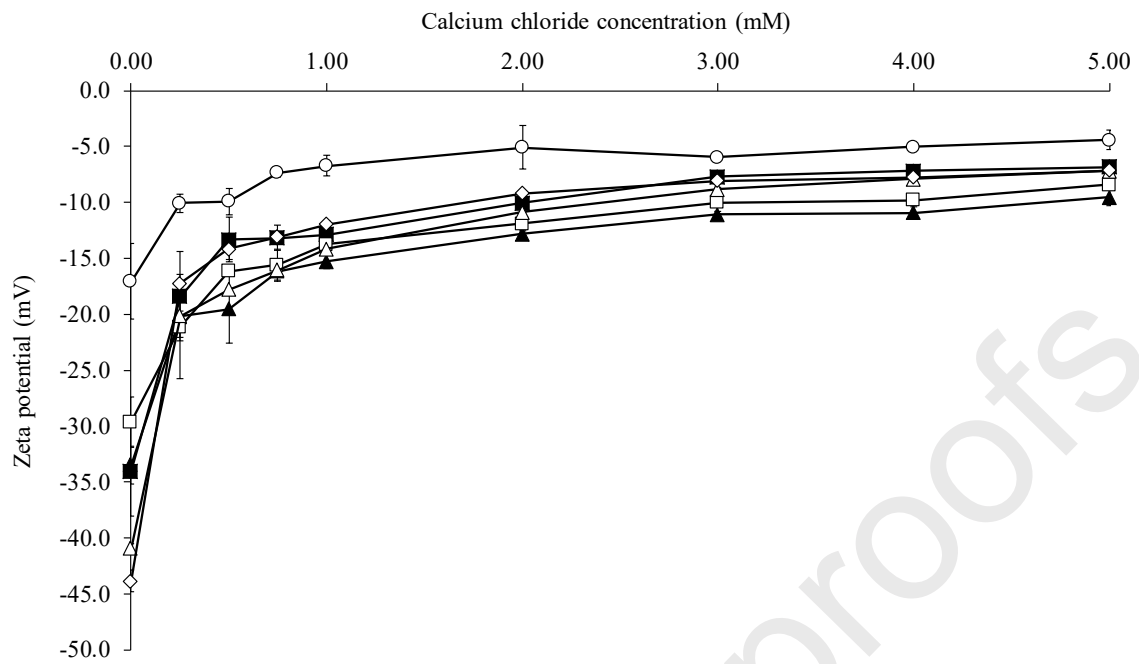
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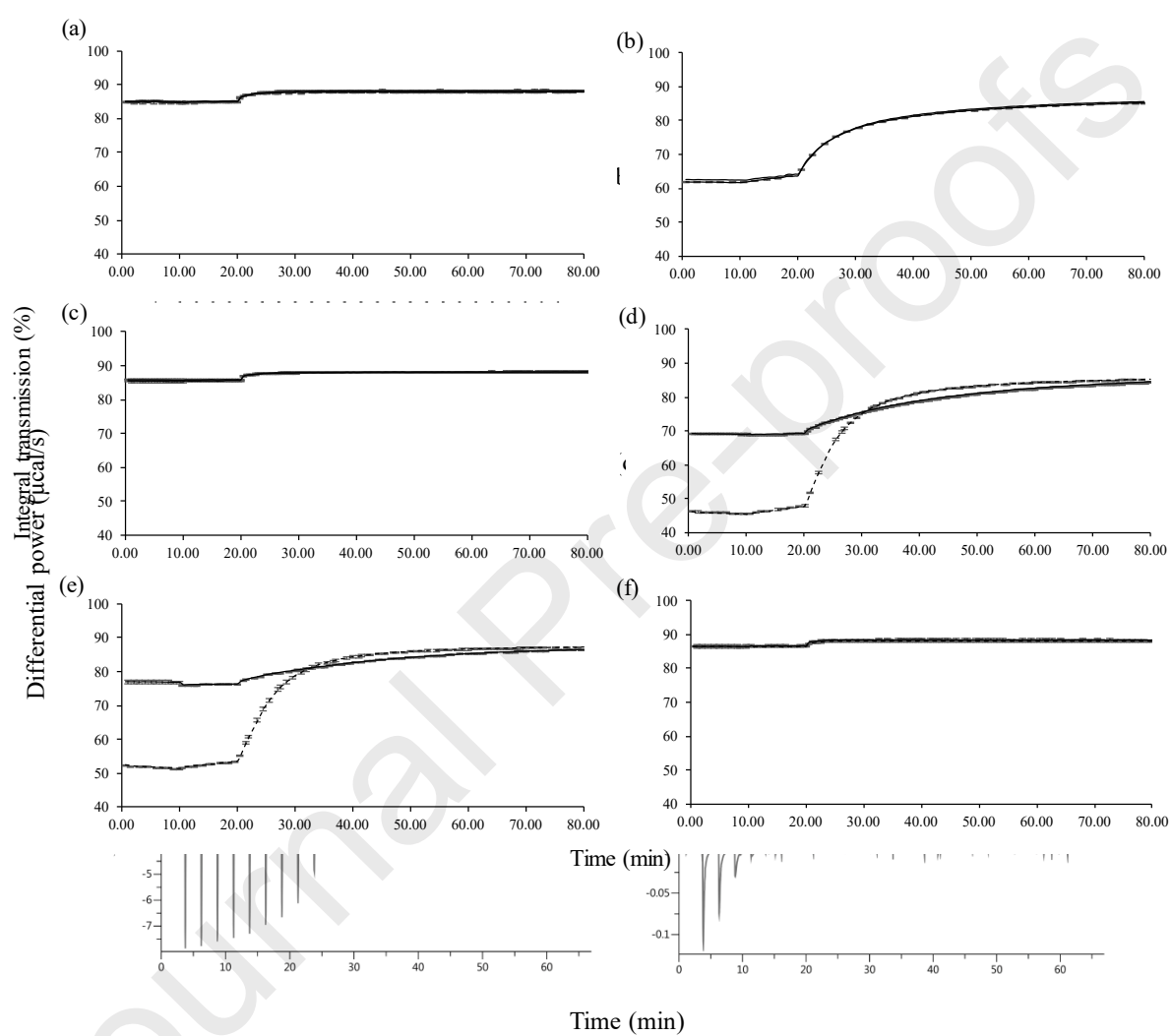
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665 Highlights



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667 • Calcium interacts with proteins in  $\alpha$ -lactalbumin-enriched WPC solutions668 • Choice of  $\alpha$ -lactalbumin enrichment approach influenced affinity for calcium

669 • Removal of phospholipids reduced calcium binding ability of WPC solutions

670 • These novel results will underpin calcium fortification of whey protein systems

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