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

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9 **Abstract**

10 In this study, three whey protein concentrate systems enriched in α -lactalbumin, produced
11 using membrane separation (LAC-M), selective precipitation (LAC-P) and ion-exchange
12 chromatography (LAC-IE), were fortified with calcium chloride (CaCl_2) at 0-5 mM and
13 changes in physicochemical properties studied. Binding of calcium (Ca^{2+}) occurred for LAC-
14 P in the range 0.00-2.00 mM, with an affinity constant (K_d) of 1.63×10^{-7} , resulting in a
15 proportion of total protein-bound calcium of 81.8% at 2 mM CaCl_2 . At 5 mM 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 α -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 α -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 (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 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 (pKa ~5.10) residues of the protein. Increasing 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, 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 α -lactalbumin (α -
61 lac), and to a lesser extent for β -lactoglobulin (β -lg) (Jeyarajah & Allen, 1994). The affinity
62 of α -lac for 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 Ca^{2+} by *apo*- α -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 α -lac: β -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 Ca^{2+} (Hebishy, Joubran, Murphy, & O'Mahony, 2019).

74 Whey protein concentrate (WPC) enriched in α -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 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 α -lac using different technological
88 approaches, with soluble calcium in the form of 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 α -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., β -
114 lactoglobulin), with α -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 α -lac by targeted adjustment of
117 pH, ionic strength and temperature. LAC-IE was manufactured using ion-exchange
118 chromatography-based separation of α -lac and β -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 α -lac content of LAC-M, LAC-P and LAC-IE powders was 28.4, 24.4 and 73.4% (w/w),
122 giving α -lac: β -lactoglobulin (β -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 α -lac contents of 20.4 and 4.36%
125 (w/w), giving α -lac: β -lg ratios of 0.24:1 and 0.28:1, respectively. The α -lac and β -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.

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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 CaCl₂, 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 CaCl₂ 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 CaCl₂ 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 CaCl₂ 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 CaCl₂
177 concentration of the dispersant, ranging from 1.330 for 0.00 mM CaCl₂ (i.e., ultrapure water)
178 to 1.332 for 5.00 mM CaCl₂, with the refractive index calculated using the Mie theory. The
179 zeta (ζ)-potential was measured at 25°C for 120 s in automatic voltage mode, and ζ -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 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 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 μL) as the
188 sample cell. The titrant was injected step-wise, in increments of 0.10 μL , 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 (ΔG), enthalpy (ΔH) 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. 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 CaCl₂) 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 CaCl₂, 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 (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 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 α -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 CaCl_2 addition for all the ingredients; the
242 relationship between $[\text{Ca}^{2+}]$ and added 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 CaCl_2 . This
245 deviation from linearity was most evident in the concentration range 0.00-2.00 mM CaCl_2
246 and these results suggest that the proteins in LAC-P and LAC-P-D had higher Ca^{2+} -binding
247 ability than those in WPI, WPC, LAC-M and LAC-IE.

248 During enrichment of α -lac from whey using selective protein precipitation (i.e.,
249 LAC-P), the α -lac protein is extensively depleted in calcium (i.e., apo- α -lac) to achieve high
250 heat-lability of α -lac. This facilitates aggregation, precipitation and selective enrichment of
251 α -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 α -lac in these protein ingredients is present mainly in the holo- α -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 CaCl_2 and $[\text{Ca}^{2+}]$. This may contrast with
258 previous studies demonstrating interactions between Ca^{2+} and α -lac in the presence of
259 phospholipid (PL) material, leading to PL-calcium- α -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 CaCl_2 addition level. The addition of 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 ΔpH
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 ΔpH of 0.36 and 0.29 were
268 measured for LAC-IE and LAC-M, respectively; whereas WPC had a ΔpH of 0.13, the
269 lowest measured ΔpH value. It is expected that the addition of soluble calcium salts (e.g.,
270 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 CaCl_2 was due to the release of hydrogen ions
275 by the proteins as a consequence of the binding of 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 ΔpH 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 CaCl_2 addition level are reported in Table 1. The measured values
282 for PSD parameters of the WPI with no added 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 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 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 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 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 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 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 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 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 CaCl₂ 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 CaCl₂ 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 α -
 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 ^a	280 ± 57.4 ^b	65.1 ± 9.29 ^a	49.1 ± 8.73 ^b	358 ± 29.6 ^{bc}	50.8 ± 8.73 ^a
	0.25	0.51 ± 0.01 ^b	216 ± 37.4 ^b	83.1 ± 0.74 ^{ab}	49.7 ± 2.75 ^c	365 ± 32.3 ^b	50.2 ± 2.75 ^a
	0.50	0.59 ± 0.01 ^c	279 ± 67.6 ^b	90.5 ± 9.84 ^b	39.3 ± 3.90 ^a	349 ± 42.3 ^{cd}	60.7 ± 3.90 ^a
	0.75	0.49 ± 0.04 ^b	273 ± 20.5 ^b	61.3 ± 3.98 ^a	46.1 ± 6.14 ^c	373 ± 22.8 ^d	53.9 ± 7.14 ^a
	1.00	0.47 ± 0.01 ^c	290 ± 19.5 ^b	77.3 ± 9.05 ^{ab}	40.3 ± 8.72 ^a	387 ± 51.7 ^{bc}	59.7 ± 10.7 ^a
	2.00	0.47 ± 0.01 ^b	259 ± 13.0 ^b	97.8 ± 12.7 ^a	43.9 ± 4.34 ^b	382 ± 19.4 ^{bc}	56.1 ± 4.38 ^a
	3.00	0.54 ± 0.01 ^d	308 ± 32.5 ^{bc}	133 ± 11.9 ^a	38.7 ± 5.25 ^{bc}	454 ± 15.2 ^d	61.3 ± 2.38 ^a
	4.00	0.51 ± 0.04 ^b	472 ± 72.1 ^{cd}	93.5 ± 9.33 ^a	38.8 ± 0.14 ^{bc}	530 ± 73.6 ^c	61.2 ± 7.07 ^a
5.00	0.76 ± 0.22 ^{bc}	638 ± 24.0 ^c	151 ± 15.6 ^b	19.4 ± 0.12 ^b	755 ± 23.9 ^c	80.6 ± 0.14 ^c	
WPC	0.00	0.24 ± 0.01 ^a	362 ± 17.0 ^b	50.5 ± 5.17 ^a	15.9 ± 2.57 ^a	409 ± 8.40 ^{cd}	84.1 ± 2.57 ^{cd}
	0.25	0.24 ± 0.01 ^a	377 ± 58.9 ^c	58.1 ± 5.36 ^a	12.8 ± 2.92 ^a	374 ± 65.5 ^b	87.2 ± 9.82 ^c
	0.50	0.32 ± 0.01 ^c	345 ± 55.2 ^c	96.1 ± 5.52 ^b	26.5 ± 0.28 ^a	395 ± 4.80 ^d	73.5 ± 0.28 ^a
	0.75	0.25 ± 0.01 ^a	255 ± 17.6 ^b	71.6 ± 2.05 ^{ab}	24.6 ± 1.62 ^a	304 ± 2.33 ^c	75.4 ± 1.62 ^c
	1.00	0.37 ± 0.02 ^{bc}	315 ± 68.2 ^b	107 ± 11.2 ^{bc}	25.6 ± 1.25 ^a	423 ± 48.3 ^c	74.4 ± 1.25 ^a
	2.00	0.23 ± 0.01 ^a	330 ± 72.8 ^{bc}	90.1 ± 8.57 ^a	30.2 ± 2.58 ^{ab}	378 ± 24.3 ^{bc}	69.8 ± 3.21 ^b
	3.00	0.24 ± 0.01 ^a	369 ± 42.2 ^c	104 ± 8.13 ^a	29.9 ± 1.40 ^b	356 ± 26.9 ^{cd}	70.1 ± 1.40 ^b
	4.00	0.27 ± 0.05 ^a	253 ± 18.7 ^{bc}	96.0 ± 8.52 ^a	26.1 ± 2.85 ^b	299 ± 15.5 ^b	73.9 ± 2.85 ^{ab}
5.00	0.25 ± 0.01 ^a	309 ± 43.3 ^b	100 ± 1.92 ^a	28.2 ± 1.82 ^c	356 ± 32.2 ^b	71.8 ± 2.82 ^b	
LAC-M	0.00	0.38 ± 0.19 ^a	264 ± 15.7 ^b	67.0 ± 7.35 ^{ab}	26.7 ± 1.34 ^a	287 ± 25.1 ^b	73.2 ± 1.34 ^{bc}
	0.25	0.29 ± 0.06 ^a	250 ± 26.2 ^b	85.9 ± 5.06 ^{ab}	34.5 ± 5.58 ^{bc}	307 ± 27.8 ^b	65.1 ± 5.58 ^{ab}
	0.50	0.25 ± 0.01 ^{ab}	241 ± 17.6 ^b	87.5 ± 2.53 ^b	39.7 ± 8.52 ^a	300 ± 9.89 ^{bc}	60.3 ± 11.5 ^a
	0.75	0.24 ± 0.01 ^a	194 ± 11.5 ^{ab}	69.8 ± 10.8 ^{ab}	31.1 ± 0.70 ^{ab}	252 ± 12.7 ^b	68.9 ± 0.72 ^{bc}
	1.00	0.27 ± 0.01 ^{ab}	258 ± 27.0 ^b	119 ± 13.1 ^c	38.9 ± 8.90 ^a	378 ± 13.2 ^{bc}	61.1 ± 8.82 ^a
	2.00	0.24 ± 0.01 ^a	225 ± 35.3 ^b	73.1 ± 4.80 ^a	43.2 ± 6.57 ^b	294 ± 25.8 ^b	56.8 ± 6.57 ^a
	3.00	0.24 ± 0.01 ^a	260 ± 14.6 ^b	76.4 ± 2.75 ^a	39.5 ± 2.12 ^c	237 ± 18.0 ^b	60.5 ± 2.12 ^a
	4.00	0.24 ± 0.01 ^a	201 ± 25.5 ^{ab}	74.9 ± 3.85 ^a	37.8 ± 4.87 ^c	225 ± 21.5 ^b	62.2 ± 4.01 ^a
5.00	0.26 ± 0.01 ^a	204 ± 61.5 ^b	105 ± 15.6 ^a	42.6 ± 0.65 ^d	357 ± 13.2 ^b	57.4 ± 0.49 ^a	
LAC-P	0.00	0.26 ± 0.01 ^a	288 ± 95.6 ^b	45.0 ± 8.87 ^a	32.2 ± 10.8 ^{ab}	334 ± 58.2 ^{bc}	66.3 ± 8.76 ^{ab}
	0.25	0.25 ± 0.01 ^a	281 ± 20.9 ^{bc}	98.0 ± 10.1 ^b	38.5 ± 1.60 ^{bc}	357 ± 29.8 ^b	61.4 ± 1.60 ^{ab}
	0.50	0.24 ± 0.01 ^a	188 ± 31.5 ^b	49.4 ± 0.80 ^a	38.3 ± 0.81 ^a	275 ± 24.6 ^b	61.7 ± 0.81 ^a
	0.75	0.24 ± 0.01 ^a	234 ± 72.1 ^{ab}	57.4 ± 5.37 ^a	39.2 ± 3.93 ^{bc}	258 ± 2.90 ^b	60.8 ± 3.95 ^{ab}
	1.00	0.24 ± 0.01 ^a	201 ± 21.5 ^b	91.7 ± 4.51 ^{bc}	36.5 ± 3.01 ^a	318 ± 16.1 ^b	63.5 ± 2.97 ^a
	2.00	0.25 ± 0.01 ^a	236 ± 28.2 ^b	93.8 ± 5.10 ^a	30.1 ± 4.51 ^{ab}	326 ± 28.2 ^{bc}	69.9 ± 1.60 ^b
	3.00	0.26 ± 0.01 ^a	249 ± 43.1 ^b	55.1 ± 3.05 ^a	40.0 ± 3.65 ^c	326 ± 31.1 ^c	60.0 ± 2.81 ^a
	4.00	0.45 ± 0.02 ^{ab}	584 ± 18.8 ^d	165 ± 6.22 ^b	20.1 ± 0.71 ^a	526 ± 35.7 ^c	79.9 ± 9.97 ^{bc}
5.00	0.50 ± 0.04 ^{ab}	638 ± 22.6 ^c	128 ± 7.25 ^{ab}	19.5 ± 2.55 ^{ab}	615 ± 40.3 ^c	80.5 ± 2.55 ^c	
LAC-P-D	0.00	0.27 ± 0.01 ^a	379 ± 21.5 ^b	91.4 ± 6.20 ^b	17.6 ± 3.30 ^a	439 ± 13.1 ^d	82.3 ± 3.30 ^{bc}
	0.25	0.27 ± 0.01 ^a	317 ± 52.5 ^{bc}	61.9 ± 8.74 ^a	30.3 ± 5.62 ^b	363 ± 32.2 ^b	69.7 ± 5.62 ^b
	0.50	0.28 ± 0.01 ^b	280 ± 31.3 ^b	83.3 ± 5.48 ^b	39.2 ± 6.07 ^a	393 ± 4.82 ^d	67.1 ± 3.02 ^a
	0.75	0.27 ± 0.01 ^a	276 ± 45.0 ^b	90.1 ± 3.39 ^b	27.6 ± 1.83 ^{ab}	389 ± 3.04 ^d	72.4 ± 1.83 ^c
	1.00	0.27 ± 0.01 ^{ab}	273 ± 24.1 ^b	66.3 ± 9.38 ^a	35.0 ± 1.64 ^a	363 ± 0.62 ^{bc}	65.0 ± 1.64 ^a
	2.00	0.27 ± 0.01 ^a	360 ± 16.7 ^c	79.9 ± 8.84 ^a	22.0 ± 5.65 ^a	400 ± 7.63 ^c	78.0 ± 5.65 ^b
	3.00	0.35 ± 0.04 ^b	760 ± 43.8 ^d	63.4 ± 5.48 ^a	9.40 ± 0.14 ^a	798 ± 11.6 ^c	90.6 ± 0.14 ^c
	4.00	0.48 ± 0.01 ^b	916 ± 84.2 ^c	151 ± 0.28 ^b	6.80 ± 0.15 ^a	929 ± 73.6 ^d	93.2 ± 0.14 ^{cd}
5.00	0.50 ± 0.04 ^{bc}	985 ± 23.2 ^d	136 ± 8.55 ^{ab}	4.40 ± 0.35 ^a	1085 ± 154 ^d	95.6 ± 0.3 ^d	
LAC-IE	0.00	0.68 ± 0.02 ^b	3.24 ± 0.89 ^a	ND	ND	3.25 ± 0.35 ^a	100 ± 0.01 ^d
	0.25	0.66 ± 0.01 ^c	3.61 ± 0.38 ^a	ND	ND	3.99 ± 0.25 ^a	100 ± 0.01 ^c
	0.50	0.43 ± 0.01 ^d	4.41 ± 0.17 ^a	ND	ND	4.60 ± 0.01 ^a	100 ± 0.01 ^b
	0.75	0.45 ± 0.04 ^b	3.87 ± 0.74 ^a	ND	ND	4.17 ± 0.61 ^a	100 ± 0.01 ^d
	1.00	0.42 ± 0.05 ^c	4.49 ± 0.46 ^a	ND	ND	4.39 ± 0.43 ^a	100 ± 0.01 ^b
	2.00	0.43 ± 0.05 ^b	4.25 ± 0.06 ^a	ND	ND	4.16 ± 0.28 ^a	100 ± 0.01 ^c
	3.00	0.45 ± 0.01 ^c	4.13 ± 0.89 ^a	ND	ND	4.71 ± 0.20 ^a	100 ± 0.01 ^d
	4.00	0.79 ± 0.08 ^c	4.13 ± 0.97 ^a	ND	ND	4.28 ± 0.24 ^a	100 ± 0.01 ^d
5.00	0.80 ± 0.04 ^c	3.87 ± 0.67 ^a	ND	ND	4.17 ± 0.18 ^a	100 ± 0.01 ^d	

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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 (ζ)-potential of whey protein solutions as a function of CaCl_2 addition level
341 is shown in Fig. 2. Prior to addition of CaCl_2 , all protein solutions displayed a net negative ζ -
342 potential. Initial ζ -potential of WPI (-34.0 mV) was in line with previous literature (Klein,
343 Aserin, Ishai, & Garti, 2010). The most negative initial ζ -potential for samples with no added
344 CaCl_2 was measured for LAC-P (-40.9 mV), while the least negative ζ -potential was
345 measured for LAC-IE (-17.0 mV). This ζ -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 α -lac (Barone, Moloney,
348 O'Regan, Kelly & O'Mahony, 2020). A plateau in ζ -potential was evident at 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 ζ -potential, with values of -8.81 and -8.08 mV, respectively.

351 The negative ζ -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 CaCl_2
354 is expected to, at least partially, shield the carboxyl groups, thereby lowering the negative ζ -
355 potential (Kulmyrzaev, Chanamai, & McClements, 2000). On increasing CaCl_2 addition, the
356 greater measured decreases in ζ -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 α -lac protein from apo- (i.e., calcium-
360 depleted) to holo- (i.e., calcium-bound) state (Wijesinha-Bettoni, Dobson, & Redfield, 2001).

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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., Ca^{2+} from 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 CaCl_2 to the protein solutions resulted in
370 negative values for Gibbs free energy (ΔG), ranging from -16.0 to -5.53 (kcal/mol),
371 suggesting that the binding of Ca^{2+} to whey protein molecules can proceed spontaneously.

372 The binding of Ca^{2+} to protein molecules in WPI, WPC and LAC-M samples resulted
373 in positive enthalpy (ΔH) and negative entropy ($-T\Delta S$) with values of 70.1, 22.2, 80.6
374 kcal/mol for ΔH and -86.3, -27.7 and -86.0 kcal/mol for $-T\Delta S$, respectively. In contrast, the
375 values for ΔH 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 $-T\Delta S$ 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 Ca^{2+} . These interactions between Ca^{2+} and proteins in LAC-P were attributed
380 to the apo-state of α -lac, which has a strong ability to bind Ca^{2+} (Permyakov & Berliner,
381 2000). This high affinity for Ca^{2+} by LAC-P in both versions was also confirmed by the
382 significantly lower ($p < 0.05$) affinity constant (K_d) for Ca^{2+} compared to the other LAC
383 samples, with values of 1.63×10^{-7} and 2.10×10^{-7} for LAC-P and LAC-P-D. Weaker binding
384 affinity for 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 α -
387 lac in this sample.

388 The negative ΔG and positive $-T\Delta S$ for both versions of the LAC-P protein system
389 indicate that the binding of 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 α -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- α -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.

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402 3.6 Calcium distribution analysis

403 The calcium content of the 1%, w/v, protein solutions with 2.00 mM 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 (ΔG), enthalpy (ΔH), 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 CaCl_2 prepared using whey protein concentrate (WPC), whey protein
 418 concentrates enriched in α -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	ΔG	ΔH (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 ± 0.55^a	70.1 ± 0.01^c	-86.3 ± 0.11^a	$1.02 \times 10^{-4}^d$	0.00 ± 0.01^a	100 ± 1.15^b	32.7 ± 2.15^c	67.4
WPC	-5.53 ± 0.01^d	22.2 ± 0.25^d	-27.7 ± 0.25^b	$8.79 \times 10^{-5}^c$	0.00 ± 0.01^a	205 ± 1.69^e	115 ± 2.47^f	43.9
LAC-M	-6.28 ± 0.01^c	80.6 ± 1.21^f	-86.0 ± 0.27^a	$2.46 \times 10^{-5}^b$	0.00 ± 0.01^a	146 ± 1.45^d	50.2 ± 1.69^e	65.6
LAC-P	-9.30 ± 0.05^b	-17.4 ± 0.05^b	8.24 ± 0.01^d	$1.63 \times 10^{-7}^a$	0.71 ± 0.01^d	97.1 ± 2.49^b	17.6 ± 1.14^a	81.8
LAC-P-D	-9.19 ± 0.02^b	-28.3 ± 0.11^a	19.1 ± 0.11^c	$2.10 \times 10^{-7}^a$	0.50 ± 0.07^c	89.2 ± 1.10^a	27.3 ± 0.53^b	69.4
LAC-IE	-6.70 ± 0.01^c	-2.02 ± 0.02^c	-4.69 ± 0.07^c	$1.21 \times 10^{-4}^c$	0.10 ± 0.01^b	110 ± 2.85^c	45.8 ± 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 CaCl₂ 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 CaCl₂ 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 CaCl₂ 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 CaCl₂ (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 CaCl₂, 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 α -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 α -
453 lactalbumin (e.g., calcium-bound or depleted) and concentrations of other macromolecules
454 (e.g., phospholipid) in the α -lac-enriched ingredients, which are in turn strongly influenced
455 by the choice of technological approach used to enrich α -lac in these ingredients. The α -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 α -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 α -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 α -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 α -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 α -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 CaCl₂ (solid line) and 5 mM CaCl₂ (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 α -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 α -lactalbumin**

645

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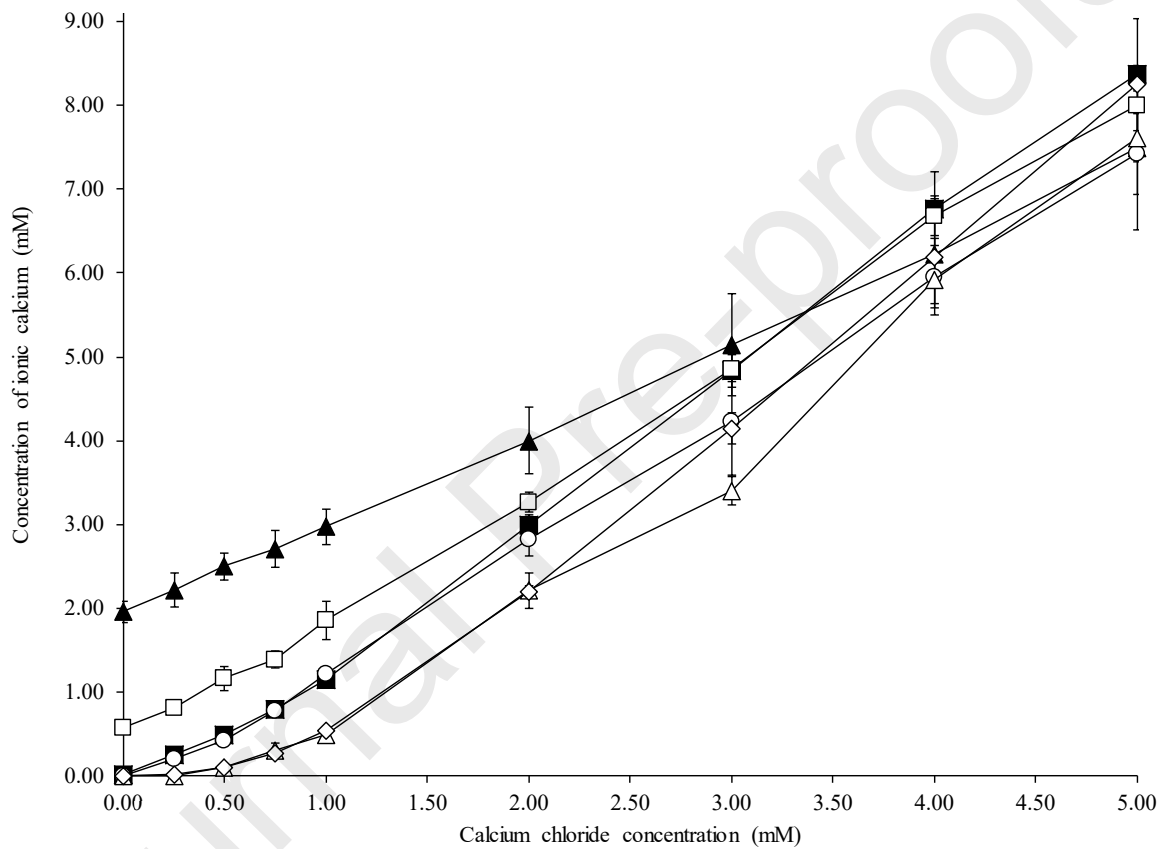
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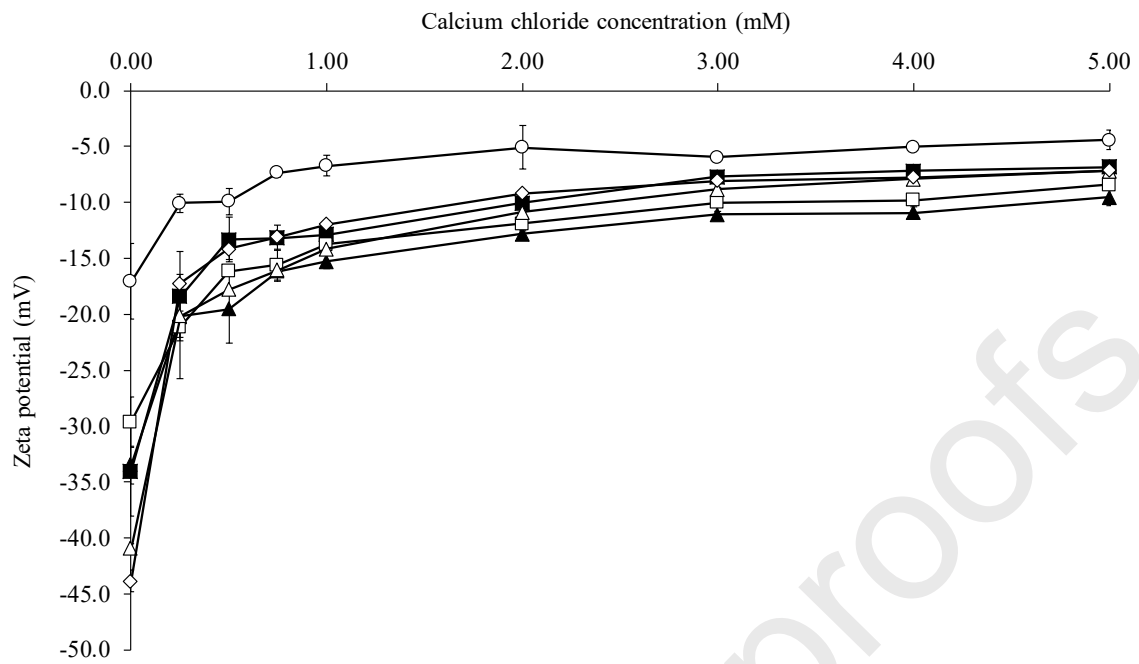
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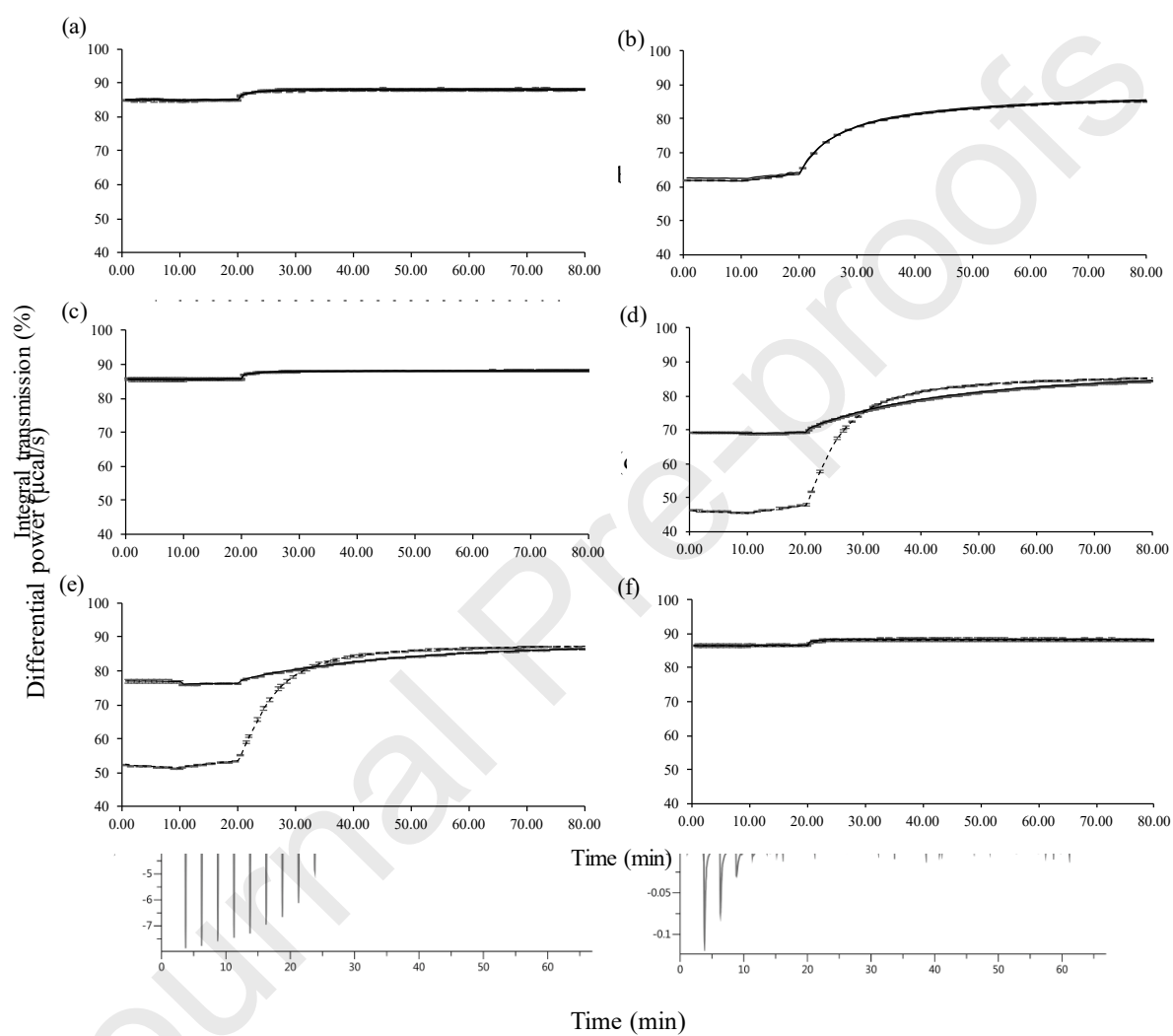
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665 Highlights



666

667 • Calcium interacts with proteins in α -lactalbumin-enriched WPC solutions668 • Choice of α -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

671