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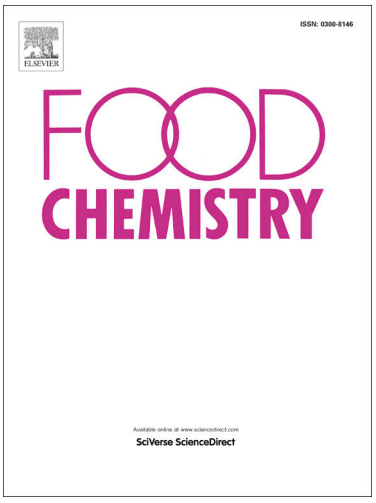

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Influence of calcium fortification on physicochemical properties of whey protein concentrate solutions enriched in α-lactalbumin

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Abstract

In this study, three whey protein concentrate systems enriched in α-lactalbumin, produced using membrane separation (LAC-M), selective precipitation (LAC-P) and ion-exchange chromatography (LAC-IE), were fortified with calcium chloride ($CaCl_2$) at 0-5 mM and changes in physicochemical properties studied. Binding of calcium (Ca^{2+}) occurred for LAC-P in the range 0.00-2.00 mM, with an affinity constant (K_d) of 1.63 x 10^{-7}, resulting in a proportion of total protein-bound calcium of 81.8% at 2 mM $CaCl_2$. At 5 mM $CaCl_2$, LAC-P had volume mean diameter (VMD) of 638 nm, while LAC-M and LAC-IE had VMD of 204 and 3.87 nm, respectively. Changes in physicochemical properties were dependent on the approach used to enrich α-lactalbumin and concentrations of other macromolecules (e.g., phospholipid). The results obtained in this study provide fundamental insights into the influence of fortification with soluble calcium salts on the physicochemical stability of next-generation whey protein ingredients enriched in α-lac.

1. Introduction

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

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

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

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

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

The influence of Ca^{2+} on the physicochemical and functional properties (e.g., heat stability, gelation and emulsification) of whey proteins has been most extensively studied using whey protein ingredients with unaltered protein profile (Keowmaneechai & McClements, 2002; Kharlamova, Nicolai, & Chassenieux, 2018; Ye & Singh, 2000). In this study, the influence of fortification of WPC enriched in α-lac using different technological approaches, with soluble calcium in the form of $CaCl_2$, on physicochemical (e.g., particle size distribution and zeta potential), thermodynamic (i.e., Gibbs free energy, enthalpy, entropy and stoichiometry) and colloidal stability of the systems was investigated. This novel work will support the development of calcium-fortified whey protein-based beverage systems with protein profiles tailored to meet specific nutritional requirements.

2. Materials and methods

2.1 Materials

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

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

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

2.2 Preparation and calcium fortification of whey protein solutions

The protein powders were reconstituted in ultra-pure water to 1% (w/v) protein, using magnetic stirring at 350 rpm for at least 2 h, followed by holding at 4°C for 18 h with continued stirring. Prior to analysis, the pH of the protein solutions was adjusted to pH 6.80 using 0.5 M potassium hydroxide or hydrochloric acid. Calcium was added in the form of $CaCl_2$, to the whey protein solutions (1%, w/v, protein) at concentrations of 0.00, 0.25, 0.50, 0.75, 1.00, 2.00, 3.00, 4.00 and 5.00 mM. Unless otherwise stated, the pH of all calcium-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 $CaCl_2$ 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 $CaCl_2$ 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.

170

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 $CaCl_2$ 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 $CaCl_2$
177 concentration of the dispersant, ranging from 1.330 for 0.00 mM $CaCl_2$ (i.e., ultrapure water)
178 to 1.332 for 5.00 mM $CaCl_2$, 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 µcal/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 (-TΔS). 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

for 25 min at 20°C in Amicon® centrifugal filter tubes (Merck Millipore, Carrigtwohill, Co. Cork, Ireland) with molecular weight cut-off of 10 kDa. Samples for AAS analysis had 24% trichloroacetic acid added in a ratio of 1:1 and allowed settle for 25 min before filtration through No. 413 filter paper (VWR International, France). The samples analysed for calcium content using AAS were the initial calcium-fortified (i.e., 2.00 mM added $CaCl_2$) protein solutions and their respective supernatants.

2.7 Accelerated colloidal stability analysis

The colloidal stability of the whey protein solutions (1%, w/v, protein, pH 6.80), with 0.00 and 5.00 mM added $CaCl_2$, was assessed using analytical centrifugation (LUMiSizer®, L.U.M. GmbH, Berlin, Germany). A three step method was used, consisting of 200 rpm from 0 to 10 min, 1000 rpm from 10 to 20 min and 4000 rpm from 20 to 80 min. Results were expressed as integral transmission of the near infrared (NIR) light as a function of centrifugation time.

2.8 Statistical data analysis

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

3. Results and discussion

3.1 Ionic calcium concentration as a function of added calcium chloride

Binding of ionic calcium (Ca^{2+}) by the different protein systems was monitored by measuring changes in ionic calcium concentration ([Ca^{2+}]) as a function of added $CaCl_2$ (Fig. 1). The initial [Ca^{2+}] (i.e., innate [Ca^{2+}]) for LAC-M and WPC was 0.58 and 1.96 mM, respectively, and were significantly higher ($p < 0.05$) than for the other samples. Differences in innate [Ca^{2+}] for LAC ingredients were expected, as it has been previously reported that the use of different α-lac enrichment technologies give rise to differences in [Ca^{2+}] between such ingredient (Barone, Moloney, O'Regan, Kelly & O'Mahony, 2020). An increase in [Ca^{2+}] was measured with increasing level of $CaCl_2$ addition for all the ingredients; the relationship between [Ca^{2+}] and added $CaCl_2$ concentration was close to linear for samples WPC, WPI, LAC-M and LAC-IE (Fig. 1), in contrast, LAC-P and LAC-P-D both displayed considerably less linear (more concave) increases in [Ca^{2+}] as a function of added $CaCl_2$. This deviation from linearity was most evident in the concentration range 0.00-2.00 mM $CaCl_2$ and these results suggest that the proteins in LAC-P and LAC-P-D had higher Ca^{2+}-binding ability than those in WPI, WPC, LAC-M and LAC-IE.

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

The LAC-P ingredient, in both original (LAC-P) and defatted (LAC-P-D) versions displayed very similar relationships between added $CaCl_2$ and [Ca^{2+}]. This may contrast with previous studies demonstrating interactions between Ca^{2+} and α-lac in the presence of phospholipid (PL) material, leading to PL-calcium-α-lac complex formation (Bo & Pawliszyn, 2006).

3.2 Titration of protein solutions with calcium chloride

The pH of the protein solutions (adjusted to an initial pH of 6.80) was measured as a function of $CaCl_2$ addition level. The addition of $CaCl_2$, in the range 0.00-5.00 mM, decreased the pH of all protein solutions. A considerable difference in pH, expressed as ΔpH (i.e., $\Delta pH = pH_{@0mM} - pH_{@5mM}$), was measured for LAC-P and LAC-P-D, with values of 0.64 and 0.61, respectively, followed by WPI (0.47). Values for ΔpH of 0.36 and 0.29 were measured for LAC-IE and LAC-M, respectively; whereas WPC had a ΔpH of 0.13, the lowest measured ΔpH value. It is expected that the addition of soluble calcium salts (e.g., $CaCl_2$) to protein solutions decreases the pH due to the release of hydrogen ions as a consequence of interactions between proteins and ions (Kharlamova, Nicolai & Chassenieux, 2018) and also due to formation of calcium phosphate, a process which results release of hydrogen ions (Lewis, 2011). Kharlamova, Nicolai & Chassenieux (2018) reported that the decrease in pH of WPI solutions on addition of $CaCl_2$ was due to the release of hydrogen ions by the proteins as a consequence of the binding of Ca^{2+} to specific sites of the protein molecules. This was also observed in the present work, especially for LAC-P, which showed the greatest ΔpH among all samples.

3.3 Particle size distribution

The data for selected particle size distribution (PSD) parameters of the whey protein solutions as a function of $CaCl_2$ addition level are reported in Table 1. The measured values for PSD parameters of the WPI with no added $CaCl_2$ (e.g., VMD of 280 nm) were similar to those reported by Loveday, Ye, Anema, & Singh (2013) for a similar protein system; the VMD values for the LAC-M and LAC-P samples with no added $CaCl_2$, ranged from 264 to 379 nm, while the VMD for the LAC-IE sample was 3.24 nm. Within samples, the VMD remained largely unchanged in the range 0.00 to 2.00 mM added $CaCl_2$, with values ranging from 4.25 to 360 nm, with LAC-IE and LAC-P-D displaying the lowest and highest VMD, respectively. At $CaCl_2$ addition levels greater than 3.00 mM, the VMD increased markedly for LAC-P-D, followed by LAC-P and WPI, with values of 916, 584 and 472 nm, respectively, at 4 mM added $CaCl_2$. A bimodal PSD (i.e., where peaks 1 and 2 correspond to small and large size material, respectively) was observed for all ingredients except LAC-IE, which had a monomodal PSD. On increasing $CaCl_2$ addition level from 0.00 to 5.00 mM, the greatest increases in volume diameter for individual particle size distribution peaks were measured for LAC-P-D, WPI and LAC-P with increases of 48.7, 131 and 184% for peak 1 and 147, 110 and 84.1% for peak 2, respectively. The WPC, LAC-M and LAC-IE samples displayed minor differences in volume diameter for individual particle size distribution peaks on increasing addition level of $CaCl_2$. The polydispersity index (PdI) values ranged from 0.23 to 0.80 for all samples, with the width of the PSD generally increasing with increasing $CaCl_2$ addition level, and the samples displaying the greatest changes in PdI were WPI, LAC-P, LAC-P-D and LAC-IE.

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

LAC ingredients (i.e., LAC-P-D) resulted in larger VMD at $CaCl_2$ addition levels greater than 2.00 mM, in comparison with the original ingredient (LAC-P). The PL components of LAC-P restricted increases in VMD, compared with LAC-P-D, when $CaCl_2$ was added at the same level. This stabilising effect of PL on the particle size in whey protein solutions can be attributed to interactions between whey proteins and PL components. The formation of PL-whey protein complexes has been reported to be mainly driven by electrostatic and hydrophobic interactions, and this complex can potentially decrease calcium-bridging between whey proteins (Alzagtat & Alli, 2002; Corredig & Dalgleish, 1996) thereby resulting in higher protein stability of calcium-fortified whey-based solutions.

330

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*	*Polydispersity index*	*Volume mean diameter*	Peak 1		Peak 2	
				Volume Diameter	*Percent of Total Area*	*Volume Diameter*	*Percent of Total Area*
	(mM)	*(PdI)*	*(nm)*	*(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^{e}	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 ± 27.5^{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^{ac}	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^{b}	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^{e}	90.6 ± 0.14^{c}
	4.00	0.48 ± 0.01^{b}	916 ± 84.2^{e}	151 ± 0.28^{b}	6.80 ± 0.15^{a}	929 ± 73.6^{d}	93.2 ± 0.14^{cd}
	5.00	0.50 ± 0.04^{ac}	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}

336

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 ($-NH_2$), whereas the carboxyl groups of
353 proteins are negatively charged ($-COO^-$); 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).
361

362 *3.5 Thermodynamic characterisation of calcium-protein interactions*

Isothermal titration calorimetry (ITC) was used in this study to better understand and quantify the thermodynamic properties of the calcium-protein interactions. ITC can be used to determine the thermodynamic properties of such interactions by measuring the heat flow produced when a ligand (i.e., Ca^{2+} from $CaCl_2$) is bound to a specific site on the protein at constant temperature. The titration thermographs and the thermodynamic constants obtained (i.e., Gibbs free energy, enthalpy, entropy, affinity constant and stoichiometry) are displayed in Fig. 3 and Table 2, respectively. The addition of $CaCl_2$ to the protein solutions resulted in negative values for Gibbs free energy (ΔG), ranging from -16.0 to -5.53 (kcal/mol), suggesting that the binding of Ca^{2+} to whey protein molecules can proceed spontaneously.

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

388 The negative ΔG and positive -TΔS 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.
401
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.

Table 2: Gibbs free energy (ΔG), enthalpy (ΔH), entropy (-TΔS), affinity constant (Kd) and stoichiometry (N) from isothermal titration calorimetry analysis of calcium-protein interactions and calcium distribution analysis between the protein-bound and free calcium in the permeate fractions after filtration through 10 kDa MWCO ultrafiltration membranes of the protein solutions added with 2 mM $CaCl_2$ prepared using whey protein concentrate (WPC), whey protein concentrates enriched in α-lactalbumin prepared using membrane filtration (LAC-M), selective protein precipitation (LAC-P), LAC-P followed by defatting (LAC-P-D), and ion-exchange (LAC-IE).

Sample	ΔG	ΔH	-TΔS	K_d	N	Calcium content of protein solution	Calcium content of permeate	Proportion of total calcium bound by protein
	———	(kcal/mol)	———	(-)	(-)	(mg/L)	(mg/L)	(%)
WPI	-16.0 ± 0.55^{a}	70.1 ± 0.01^{e}	-86.3 ± 0.11^{a}	1.02 x 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 x 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 x 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 x 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^{e}	2.10 x 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 x 10^{-4} ^{e}	0.10 ± 0.01^{b}	110 ± 2.85^{c}	45.8 ± 2.42^{d}	58.6

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

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

3.7 Accelerated suspension stability

Analytical centrifugation was used to evaluate the optical properties and suspension stability of the 1% protein solutions with 0.00 and 5.00 mM $CaCl_2$ added. Different initial (i.e., 0 min) optical properties of the ingredients were observed (Fig. 4), with WPC having the lowest transmission (62.0%), while LAC-IE had the highest transmission (86.5%). Addition of 5.00 mM $CaCl_2$ resulted in minimal changes in integral transmission of the samples, except for LAC-P and LAC-P-D in which significantly lower transmission (49.4 and 46.1% for LAC-P and LAC-P-D, respectively) was measured when compared to their counterparts with 0.00 mM $CaCl_2$ addition. Centrifugation resulted in slight clarification (i.e., higher integral transmission) for all ingredients, with greater clarification observed for LAC-P and LAC-P-D at 5.00 mM $CaCl_2$ (Fig. 5). This physical instability (i.e., clarification on centrifugation) is in agreement with the PSD analysis presented earlier, as at 5.00 mM added $CaCl_2$, the VMD of LAC-P increased, which influenced the optical (i.e., lower transmission) and colloidal properties.

Conclusion

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

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Figure 1: Concentration of ionic calcium (mM) as a function of calcium chloride concentration (mM) for 1% protein solutions at pH 6.80 prepared from whey protein isolate (WPI; —■—), whey protein 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 $CaCl_2$ (solid line) and 5 mM $CaCl_2$ (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 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**

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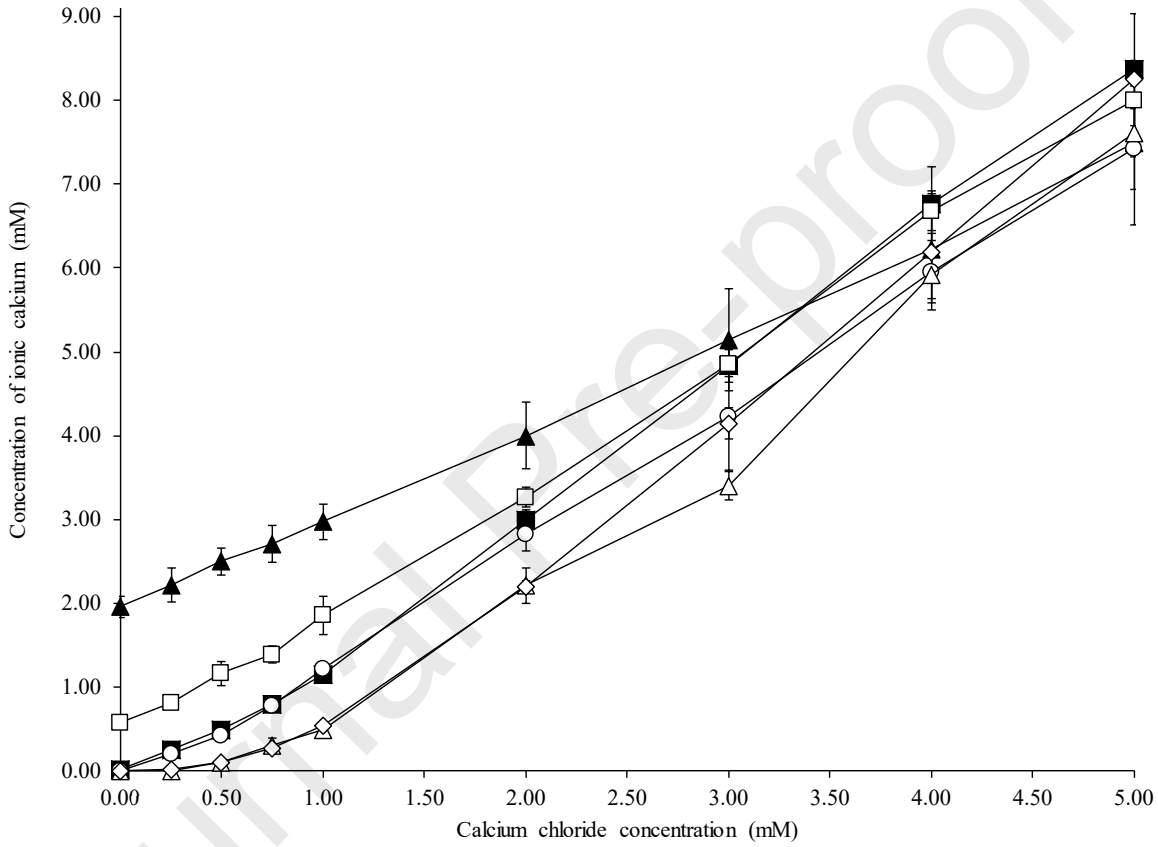

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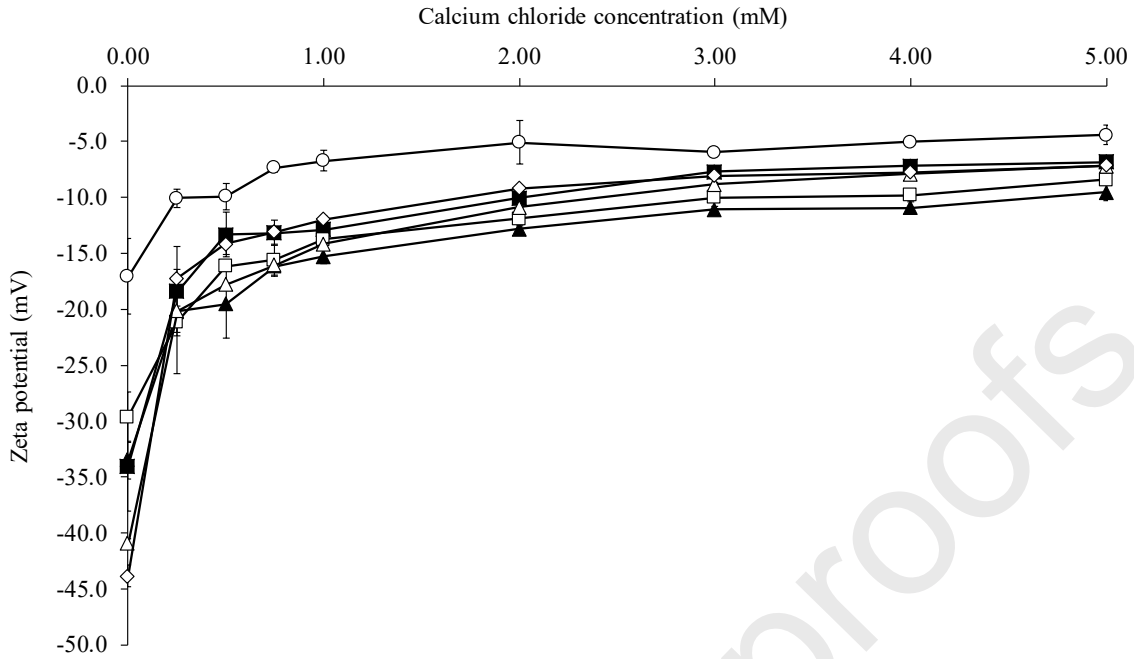

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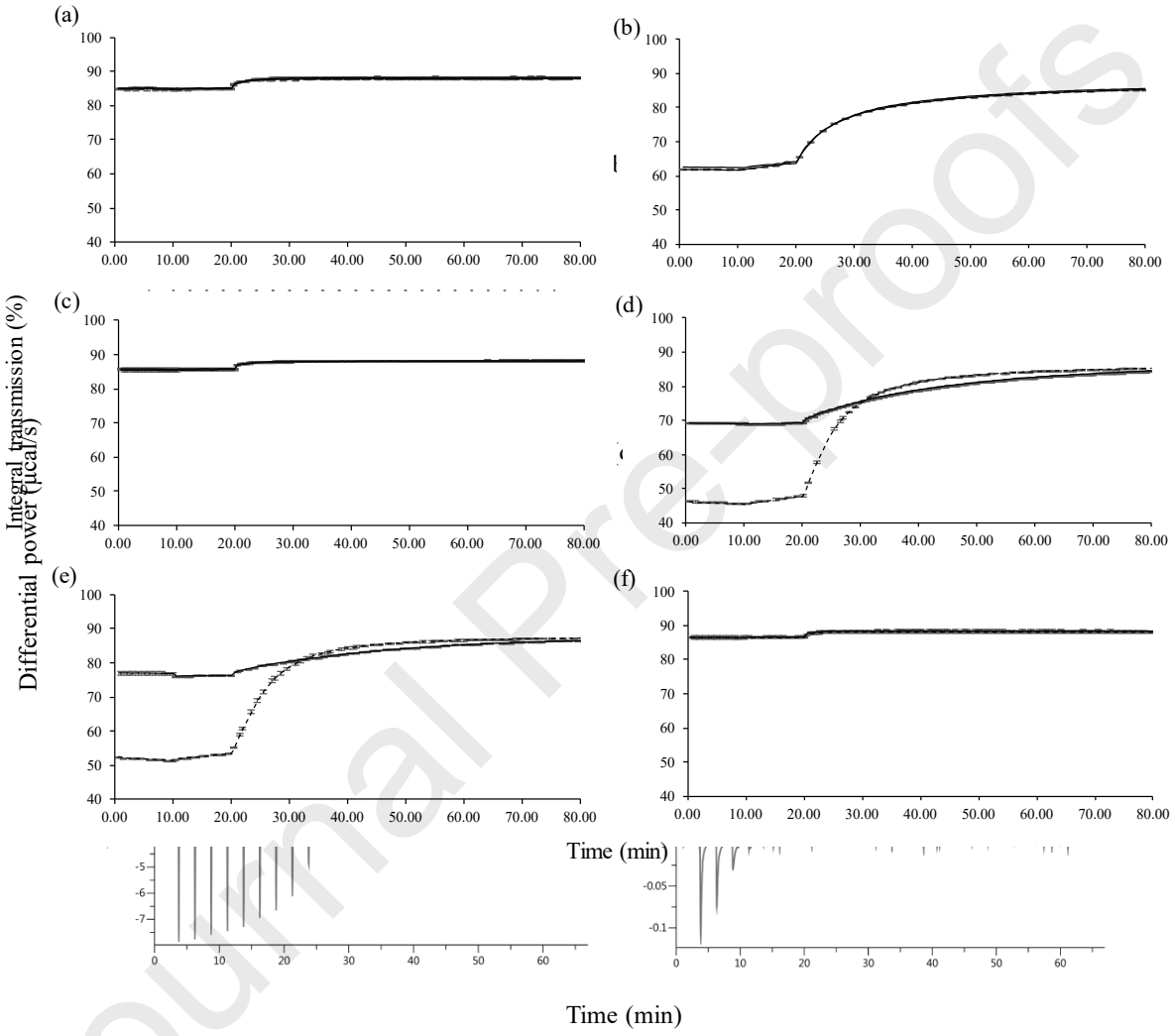
665 Highlights



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667 • Calcium interacts with proteins in α-lactalbumin-enriched WPC solutions

668 • 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

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