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1 **The Use of High Performance Liquid Chromatography for the**
2 **Characterisation of the Unfolding and Aggregation of Dairy Proteins**

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7 Running head: HPLC for dairy proteins

8

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9 **Abstract**

10 High performance liquid chromatography (HPLC) is routinely used to identify and characterise
11 proteins. HPLC can to understand protein aggregation processes in dairy products, which are
12 induced by common industrial processing steps such as heat treatment. In this chapter, three
13 complimentary chromatographic methods are described, which are based on the principles of size
14 exclusion and reversed-phase chromatography. These methods are used to determine the degree of
15 denaturation and aggregation of proteins, and estimate the molecular weight of these aggregates.

16 Key words: HPLC, proteins, denaturation, aggregation, reversed-phase chromatography, size
17 exclusion chromatography

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19 1. Introduction

20 High performance liquid chromatography, abbreviated HPLC, is a routine technique developed in
21 the 1960s to purify and analyse polar molecules with a high molecular weight in less than one hour
22 (1). Thanks to significant improvements in chromatography matrices and the packing of columns,
23 HPLC can now be used as a tool to analyse peptides, proteins and biopolymers with great accuracy
24 and reproducibility. The characterisation of proteins, in particular protein unfolding and aggregation,
25 is of great importance in the field of biochemistry, but also widely used in food and biomaterial
26 sciences. This chapter describes the use of HPLC in dairy chemistry, in particular for the
27 characterisation of the state of dairy proteins (native, unfolded, aggregated) due to common process-
28 induced changes during food production (e.g. heat treatment, high pressure, concentration,
29 dehydration, change in acidity and ionic strength etc.). Chromatographic separation is based on the
30 size (gel permeation or size exclusion chromatography, SEC-HPLC) or polarity (reversed-phase
31 chromatography, RP-HPLC) of the protein material. By combining these methods, a detailed
32 characterisation of the extent of protein denaturation and aggregation is possible (2-4). The
33 chromatography results contribute to the overall kinetic and structural understanding of heat-induced
34 changes in the structure of dairy proteins, which is of high scientific and industrial interest. A
35 summary of other methods for the quantification of dairy proteins can be found elsewhere (5).

36 Three complimentary HPLC methods are described in this chapter:

37 Method 1: RP-HPLC method for the total quantification of dairy proteins (caseins and whey
38 proteins, native and aggregated), based on a method by Visser *et al.* (6). It allows the quantification
39 of individual proteins, including those in aggregates. Sample treatment involves the disruption of the
40 intermolecular disulphide bonds and non-covalent interactions by β -mercaptoethanol and urea (7).

41 Method 2: RP-HPLC method for the quantification of native whey proteins based on a method by
42 Beyer *et al.* (8,9). Sample treatment involves the isoelectric precipitation and removal of denatured
43 whey proteins. The degree of protein denaturation can be calculated from the difference between the

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44 total amount of proteins and that of the native proteins. This is only applicable for whey proteins,
45 which can unfold and denature because of their globular structure.

46 Method 3: SEC-HPLC method for the estimation of the molecular weight of the proteins and protein
47 aggregates. The method is suitable for molecular weight ranges between approximately 10^4 and
48 5×10^5 Da, depending on the choice of chromatography column.

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50 2. Materials

51 All solutions should be prepared using ultrapure water, such as Milli-Q® water and analytical or, if
52 available, HPLC-grade reagents. All solutions containing acetonitrile (ACN) or trifluoroacetic acid
53 (TFA) must be prepared in a fume hood using the correct PPE (lab coat, lab goggles and appropriate
54 gloves).

55 *A. Preparation of Mobile Phase A (for method 1), 10% (v/v) acetonitrile (ACN) + 0.1% (v/v)*
56 *trifluoroacetic acid (TFA) in water.* Carefully pour 200 mL of ACN into a 2 L volumetric flask. Add
57 around 1,500 mL of water and 2 mL of TFA (*see Note 1*). Invert the solution to thoroughly mix the
58 organic phase and water. Fill up to 2 L with water. Rinse a filtration vessel and a 2 L glass bottle
59 with a small amount of the filtered mobile phase and return it to the unfiltered buffer. Vacuum filter
60 the mobile phase with a 0.45 µm pore size hydrophilic filter (e.g. Durapore hydrophilic PVDF
61 membrane filter type, Merck Millipore). Store at room temperature (*see Note 2*).

62 *B. Preparation of Mobile Phase B (for method 1 and 2), 90% (v/v) ACN + 0.1% (v/v) trifluoroacetic*
63 *acid (TFA) in water.* First add 200 mL of water to a 2 L volumetric flask (*see Note 3*). Carefully add
64 2 mL of TFA and slowly add ACN up to 10 cm below the fill line. Invert the solution to mix
65 thoroughly. Wait 20 min to fully equilibrate and fill up to the 2 L mark (*see Note 4*). Rinse a
66 filtration vessel and a 2 L glass bottle with a small amount of the filtered mobile phase and return it
67 to the unfiltered buffer. Vacuum filter the mobile phase with a 0.45 µm pore size hydrophobic filter
68 (e.g Durapore hydrophobic PVDF membrane filter type, Merck Millipore). Store at room
69 temperature (*see Note 2*).

70 *C. Preparation of Mobile Phase A (for method 2), 0.1% (v/v) trifluoroacetic acid (TFA) in water.*
71 Mix approximately 1,800 mL of water and 2 mL of TFA (*see Note 1*). Make up to 2 L with water.
72 Invert the solution to thoroughly mix the water and TFA. Rinse a filtration vessel and a 2 L glass
73 bottle with a small amount of the filtered mobile phase and return it to the unfiltered buffer. Vacuum

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74 filter the mobile phase with a 0.45 μm pore size hydrophilic filter (e.g. Durapore hydrophilic PVDF
75 membrane filter type, Merck Millipore). Store at room temperature (*see Note 5*).

76 D. *Preparation of the mobile phase (for method 3), 20 mM sodium phosphate, pH 7.0.* Prepare 1 L of
77 20 mM monobasic sodium phosphate (NaH_2PO_4) and 1 L of 20 mM dibasic sodium phosphate
78 (Na_2HPO_4). Add solid sodium azide to reach a concentration of 0.05% (w/v) in both solutions to
79 inhibit undesirable microbial growth. Add 900 mL of 20 mM dibasic sodium phosphate to a 2 L
80 beaker and stir continuously. Slowly add 20 mM monobasic sodium phosphate until pH 7.0 is
81 reached. Rinse a filtration vessel and a 2 L glass bottle with a small amount of the filtered mobile
82 phase and return it to the unfiltered buffer. Vacuum filter the mobile phase through a 0.45 μm pore
83 size hydrophilic filter (e.g. Durapore hydrophilic PVDF membrane filter type, Merck Millipore).
84 Store at room temperature (*see Note 6*).

85 E. *0.1 M Sodium acetate/acetic acid buffer pH 4.6.* Prepare 0.1 M of sodium acetate with water in
86 500 mL volumetric flask, in the fume hood. Prepare 0.1 M acetic acid with water in a 500 mL
87 volumetric flask, in the fume hood. Transfer 400 mL of 0.1 M acetic acid solution to a 1 L beaker
88 and slowly add 0.1 M sodium acetate until pH 4.6 is reached. Store at room temperature (*see Note*
89 *7*).

90 F. *Denaturing sample buffer: 7 M urea + 20 mM bis-tris propane, pH 7.5.* Weigh 42 g of urea ($M_w =$
91 60.06 g/mol) and 0.56 g of bis-tris propane (1,3-bispropane, $M_w = 282.334$ g/mol) in a glass beaker
92 with 80 mL of water (*see Note 8*). Stir and heat gently to aid dissolution. Adjust the pH to 7.5 using
93 0.1 M HCl or NaOH. Transfer to a 100 mL volumetric flask and rinse the transfer funnel with a
94 small amount of water. Add water to 100 mL. Invert several times to mix thoroughly. (*see Note 9*).

95 G. *Molecular weight standards.* The molecular weight standards (*see Table 1*) are prepared in water.
96 (Table 1 near here)

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98 **3. Methods**

99 **3.1 Method 1: Quantification of dairy proteins using RP-HPLC**

100 **3.1.1 Sample preparation for dairy protein quantification**

101 Caseins exist in milk as large, colloidal particles (casein micelles, mean diameter \approx 150 nm)
102 suspended in the aqueous milk serum, the latter containing whey proteins. Caseins associate via non-
103 covalent interactions (**10**). In contrast to this, native whey proteins are in monomeric or dimeric
104 form. Upon heating, whey proteins and caseins can associate via covalent disulphide bonds and
105 other non-covalent interactions. For chromatographic separation, the proteins need to be dissociated
106 and fully denatured prior injection onto the column. The non-covalent interactions and disulphide
107 bonds can be disrupted by pre-treating samples with urea and β -mercaptoethanol. In this method, the
108 samples are mixed with the denaturing sample buffer in a ratio of 1:20 (*see Note 10*).

- 109 1. In order to reach the desired final concentration of 0.2% (w/v) of proteins (*see Note 11*),
110 standardise the protein sample to 3.5-4% (w/v) protein. The protein standards, native whey proteins
111 and caseins are prepared in water.
- 112 2. Transfer the volume of sample buffer needed for a volume ratio sample:buffer of 1:20, to a
113 polypropylene tube and add 50 μ L of β -mercaptoethanol for every 10 mL of sample buffer.
- 114 3. Add 200 μ L of each sample to 3.8 mL of urea and β -mercaptoethanol mixture. Vortex the
115 samples. Leave at room temperature for 1 h and invert every 15 min (*see Note 12*).
- 116 4. Filter the samples through a 0.22 μ m low protein binding and hydrophilic syringe filter (e.g.
117 PVDF membrane filter type) into the HPLC vials. Fill to the neck.

118 **3.1.2 HPLC system**

119 The method requires a HPLC separation module with a UV/visible detector and the corresponding
120 software for data analysis.

121 The results were obtained here using a Poroshell 300SB-C18 column measuring 2.1 \times 75 mm from
122 Agilent (Santa Clara, CA, USA).

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123 One chromatographic run takes 35 min per sample at a flow rate of 0.5 mL/min. The injection
124 volume is 5 μ L. The column temperature is set at 35°C.

125 **3.1.3 HPLC run and analysis of the elution profiles**

- 126 1. Equilibrate the column with 2 to 5 column volumes of a mobile phase mixture of 74%
127 Mobile Phase A and 26% of mobile phase B at a flow rate of 0.5 mL/min. The absorbance is
128 recorded at 214 and 280 nm (see **Note 13**). After equilibration, the absorbance should be constant
129 and changes in absorption close to $\pm 10^{-5}$ AU; extend the equilibration if necessary.
- 130 2. Set up the HPLC instrument method to run the gradient detailed in **Table 2**, at a flow rate of
131 0.5 mL/min and at a column temperature of 35°C.
- 132 3. Inject 5 μ l of a blank (water or mobile phase) at the beginning and end of each set of
133 samples to verify a clean baseline. Inject 5 μ L of the samples and the standards. The order for the
134 injection should follow an increasing protein concentration to reduce the risk of cross-contamination.
- 135 4. Compare the elution time of the standards to the elution time of the unknown proteins to
136 identify the peaks. Use the software functions to integrate the individual peaks and deduce the
137 protein content of each protein from a calibration curve for each protein standard. Anticipated
138 elution profiles of caseins and whey proteins at 214 nm are shown in **Figure 1**.

139 (Table 2 near here)

140 (Figure 1 near here)

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142 **3.2 Method 2: Quantification of whey protein denaturation by RP-HPLC**

143 **3.2.1 Sample preparation**

- 144 1. Dilute the protein standards in ultrapure water.
- 145 2. Dilute the protein samples in sodium acetate/acetic acid buffer at pH 4.6 to reach a protein
146 concentration of 0.25% (w/v) (*see Note 11*). Separate the isoelectric precipitate by centrifugation at
147 14,000×g for 30 min at room temperature (*see Note 14*).
- 148 3. If dilution of the supernatant is necessary, dilute in a mixture of 80% Mobile Phase A and
149 20% of mobile phase B (*see Note 15*).
- 150 4. Discard the pellet and filter the supernatant and the protein standards through 0.45 µm low
151 protein binding and hydrophilic syringe filter (e.g. PES membrane filter type, Sartorius, Göttingen,
152 Germany) directly into the HPLC vials. Fill to the neck of the vial.

153 **3.2.2 HPLC system**

154 The method requires a HPLC separation module with a UV/visible detector and the corresponding
155 software for data analysis.

156 The results were obtained using a C5 PolymerX RP1 column (*see Note 16*) measuring 150×4.6 mm
157 from Phenomenex (Torrance, CA, USA). One chromatographic run takes 45 min per sample at a
158 flow rate of 1 mL/min. The injection volume is 20 µL. The column temperature is set at 28°C.

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160 **3.2.3 HPLC run and analysis of the elution profiles**

- 161 1. Equilibrate the column with 2 to 5 column volumes of a mobile phase mixture of 80%
162 Mobile Phase A and 20% of mobile phase B with a flow rate of 1 mL/min. The absorbance is
163 recorded at 214 and 280 nm (*see Note 13*). After equilibration, the absorbance should be constant
164 and changes in absorption close to $\pm 10^{-5}$ AU; extend the equilibration if necessary.
- 165 2. Set up the HPLC instrument method to run the gradient detailed in **Table 3**, at a flow rate of
166 1 mL/min and at a temperature of 28°C.
- 167 3. Inject 20 μ l of a blank (water or mobile phase) at the beginning and end of each set of
168 samples to verify a clean baseline. Inject 20 μ l of the samples and the standards. The order for the
169 injection should follow an increasing protein concentration to reduce the risk of cross-contamination.
- 170 4. Compare the elution time of the standards to the elution time of the unknown proteins to
171 identify the peak. Use the software functions to integrate the individual peaks and deduce the protein
172 content of each protein from a calibration curve for each protein standard. The amount of denatured
173 protein is calculated as the difference between the initial amount of non-heated protein samples and
174 the residual amount after heating, both determined by this method. Alternatively, the total (native +
175 denatured) amount of protein can be determined by the method described in section 3.1.
- 176 5. Anticipated elution profiles of caseinomacropptide (CMP), α -lactalbumin (α -la) and β -
177 lactoglobulin (β -lg) at 280 nm and 214 nm are shown in **Figure 2**.

178 (Table 3 near here)

179 (Figure 2 near here)

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187 **3.3 Method 3: Determination of the degree of protein aggregation by SEC-HPLC system**

188 Two columns in series, TSK Gel G2000SW_{XL} and TSK Gel G3000SW_{XL} (Tosoh Bioscience GmbH,
189 Griesheim, Germany) are used, preceded by a guard column to prevent potential column blockage.
190 The dimensions of both columns are 7.8 x 300 mm (*see Note 17*).

191 The method requires a HPLC separation module, a UV/visible detector and the corresponding
192 software for the elution analysis. The flow rate is 0.5 mL/min with an isocratic gradient of 20 mM
193 sodium phosphate (pH 7.0). The total duration of the run is 60 min per sample. The injection volume
194 is 20 µl. The column should remain at room temperature without the use of a column oven (*see Note*
195 **18**).

196 **3.3.1 HPLC run and analysis of the elution profiles**

197 The samples are standardised to 0.25% (w/v) protein in water (*see Note 11*) and filtered through
198 0.45 µm hydrophilic syringe filters with a low protein binding profile (e.g. PES membrane filter
199 type, Sartorius, Göttingen, Germany). The molecular weight standards were prepared as described in
200 section 2.

- 201 1. Equilibrate the column with 2 column volumes of 20 mM of sodium phosphate (pH 7.0;
202 0.05%, w/v, sodium azide) buffer at a flow rate of 0.5 mL/min. The absorbance is recorded at 214
203 and 280 nm (*see Note 13*). After equilibration, the absorbance at 280 nm should be constant and
204 changes in absorption close to $\pm 10^{-5}$ AU; extend the equilibration if necessary.
- 205 2. Set up the HPLC instrument method to run an isocratic gradient of 20 mM sodium
206 phosphate (pH 7.0) at a flow rate of 0.5 mL/min.
- 207 3. Inject 20 µl of a blank (water or mobile phase) at the beginning and end of each set of
208 samples to verify a clean baseline. Then, inject 20 µl of the samples and the standards. The order for

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209 the injection should follow an increasing protein concentration to reduce the risk of cross-
210 contamination.

211 4. Calculate the partition coefficient K_{av} of the standards.

212 K_{av} is expressed as:

213 $K_{av}=(V_e-V_0)/(V_c-V_0)$,

214 where V_e is the volume at which the peak was eluted, V_0 is the exclusion volume and V_c is the
215 volume of the column. V_0 is the elution volume of the blue dextran.

216 Plot K_{av} against the logarithm of the molecular weight of the standards.

217 Calculate K_{av} of the sample peaks and deduce the molecular weight of the proteins and aggregates
218 using the calibration curve.

219 5. An anticipated elution profile of, α -lactalbumin (α -la), β -lactoglobulin (β -lg) and heat-
220 induced aggregates of whey proteins at 280 nm is shown in **Figure 3**.

221 (Figure 3 near here)

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223 **4. Notes**

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225 1. TFA (trifluoroacetic acid) is an anionic ion-pairing agent interacting with the stationary
226 phase of the column and with the positively charged portions of hydrophilic proteins and peptides,
227 affecting their retention time. TFA is also UV-transparent, which makes it a suitable additive to
228 HPLC solvents. TFA is very volatile; it is recommended to first add the acetonitrile or water and
229 then the TFA when preparing the mobile phase to avoid loss. Due to its acute toxicity, it must be
230 handled in the fume hood while wearing the appropriate PPE.

231 2. Buffers containing acetonitrile are very stable and no microbial growth is expected. Thus,
232 the addition of sodium azide is not necessary and the mobile phase can be used for up to one year in
233 an air-tight bottle.

234 3. The addition of 10% (v/v) water reduces the differences in viscosity of the two mobile
235 phases (organic and aqueous) and improves mixing in the HPLC separation module before entering
236 the column.

237 4. Mixing acetonitrile with water causes an endothermic reaction and a cooling of the mobile
238 phase can be observed. Waiting for the solution to reach room temperature minimises error in the
239 volume adjustment, thereby improving reproducibility.

240 5. The addition of TFA reduces the pH and also limits the risk of microbial growth. Thus, the
241 aqueous mobile phase containing TFA can be used for several months after preparation; the addition
242 of sodium azide is not necessary.

243 6. Sodium phosphate buffer containing 0.05% (w/v) sodium azide can be used for up to one
244 month after preparation.

245 7. Sodium acetate/acetic acid buffer can be used within a few months due to the low pH of the
246 buffer.

247 8. Guanidine hydrochloride (6 M) and dithiotreitol (19.5 mM) can be used as denaturing and
248 reducing agent instead of urea and β -mercaptoethanol to improve the separation of some of the
249 proteins (*11,12*).

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250 9. The buffer should be freshly prepared and cannot be stored for long due to the high
251 concentration of urea. The prolonged storage of urea leads to the formation of crystals.

252 10. In case of samples with a low protein concentration, a ratio sample: denaturing buffer of 1:4
253 can be used.

254 11. The adjustment of the protein concentration to around 0.25% (w/v) is an indicative figure.
255 We observed a reasonable separation at this protein concentration, but this can be adjusted if
256 necessary.

257 12. Urea denatures the proteins by disrupting hydrogen bonds. This requires a high
258 concentration of urea. Without heating, β -mercaptoethanol requires more time to reduce the
259 disulphide bonds of the proteins.

260 13. The choice of the adequate wavelength of detection can be made prior chromatographic
261 separation by measuring an absorption spectrum of the sample with a UV/vis spectrophotometer.
262 Most proteins and peptides contain aromatic amino acids that absorb at 280 nm. For some
263 polypeptides, such as caseinomacropptide, and generally shorter peptides, a detection wavelength
264 of 214 nm is recommended, which corresponds to the absorption by the peptide bonds.

265 14. Centrifugation of the proteins at pH 4.6 allows the isoelectric precipitation of aggregated
266 and denatured whey proteins. The centrifugation speed and duration are chosen to minimise the loss
267 of native proteins in the pellet.

268 15. Using the original composition of the mobile phase for diluting the sample prevents a
269 potential precipitation of the proteins in the column during the HPLC run. Precipitation of proteins
270 in a chromatography column can cause irreversible blockages and damage to the stationary phase.
271 Measuring the protein content before and after filtration is recommended to verify their solubility.
272 Native proteins can be rehydrated in water unless precipitation is expected in the mobile phase.

273 16. C4 columns (**13**) or a PLRP-S column from Latek (Eppelheim, Germany) can also be used
274 as an alternative (**14**). The elution gradient and acetonitrile/water ratio of the mobile phases were, in
275 both case, slightly modified.

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276 17. The use of two columns in series increases the number of theoretical plates and thus the
277 quality of the separation. Using two G2000 columns of 300 mm or one G2000 column of 600 mm
278 gives good results to analyse the disappearance of native whey proteins and appearance of soluble
279 aggregated proteins during heat-treatment. The exclusion volume of the G3000 column corresponds
280 to a higher molecular weight (5×10^5 Da) and allows the detection of larger aggregates. The OHpak
281 SB-806 HQ-type column from Shodex (Tokyo, Japan), can separate even larger aggregates
282 (exclusion volume corresponding to 2×10^7 Da). It is noteworthy that these SEC columns are very
283 stable if treated with care.

284 18. The results obtained by SEC-HPLC are less sensitive to temperature variations because
285 hydrophobic interactions with the stationary phase are minimal, contrary to RP-HPLC.

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345 **Figure captions**

346 **Figure 1:** Anticipated results (method 1) of elution of κ -casein (κ -CN), α_{s2} -casein (α_{s2} -CN), α_{s1} -
347 casein (α_{s1} -CN), β -casein (β -CN), α -lactalbumin (α -la), β lactoglobulin (β -lg A and B) from skim
348 milk on a Poroshell 300SB-C18 column (Agilent, Santa Clara, CA, USA) at flow rate of 0.5
349 mL/min. The mobile phase A was 10% (v/v) acetonitrile (ACN) + 0.1% (v/v) trifluoroacetic acid
350 (TFA) in water and the mobile phase B was 90% (v/v) ACN + 0.1% (v/v) TFA in water.

351 **Figure 2:** Anticipated chromatograms (method 2) of native caseinomacropeptide (CMP), α -
352 lactalbumin (α -la) and β -lactoglobulin (β -lg A and B) on a C5 PolymerX RP1 column
353 (Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL/min, detected at 280 nm (A) and 214 nm
354 (B). The mobile phase A was 0.1% TFA (v/v) in water and the mobile phase B was 90% (v/v) ACN
355 + 0.1% (v/v) TFA in water.

356 **Figure 3:** Chromatograms (method 3) showing the typical profile of (A) the whey proteins α -
357 lactalbumin (α -la) and β -lactoglobulin (β -lg), and (B) heat-induced aggregates of α -lactalbumin and
358 β -lactoglobulin on TSK Gel G2000SW_{XL} and TSK Gel G3000SW_{XL} in series (Tosoh Bioscience
359 GmbH, Griesheim, Germany) eluted at a flow rate of 0.5 mL/min by SEC-HPLC. The mobile phase
360 was 20 mM sodium phosphate (pH 7.0).

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362 **Table captions**

363 **Table 1**

364 Protein standards for SEC-HPLC of protein aggregates on a TSK Gel G2000SW_{XL} and a TSK Gel
365 G3000SW_{XL} in series (Tosoh Bioscience GmbH, Griesheim, Germany).

366 **Table 2**

367 Gradient of elution for the separation of caseins and whey proteins on a Poroshell 300SB-C18
368 column (Agilent, Santa Clara, CA, USA). Solvent A: 10% (v/v) ACN in 0.1% (v/v) TFA; solvent B:
369 90% (v/v) ACN in 0.1% (v/v) TFA.

370 **Table 3**

371 Gradient of elution for the separation of native whey proteins on a C5 PolymerX RP1 column
372 (Phenomenex, Torrance, CA, USA). Solvent A: 0.1% (v/v) TFA; solvent B: 90% (v/v) ACN in 0.1%
373 (v/v) TFA.

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375 **Table 1**

| Protein | Molecular weight (Da) |
|------------------------|-----------------------|
| Blue dextran | >2,000,000 |
| Thyroglobulin | 669,000 |
| Ferritin | 440,000 |
| Aldolase | 158,000 |
| Bovine serum albumin | 66,267 |
| β -lactoglobulin | 18,362 |
| α -lactalbumin | 14,174 |

376 Proteins can be purchased as a high molecular weight kit (GE Healthcare, Little Chalfont, U.K.) in
 377 addition to bovine serum albumin, β -lactoglobulin and α -lactalbumin (Sigma Aldrich, St. Louis,
 378 MO, USA).

379 **Table 2**

| Time (min) | % A | % B |
|------------|-----|-----|
| 0.0 | 74 | 26 |
| 10.0 | 63 | 37 |
| 23.0 | 55 | 45 |
| 26.0 | 0 | 100 |
| 29.5 | 0 | 100 |
| 32.5 | 74 | 26 |
| 35.0 | 74 | 26 |

380

381 **Table 3**

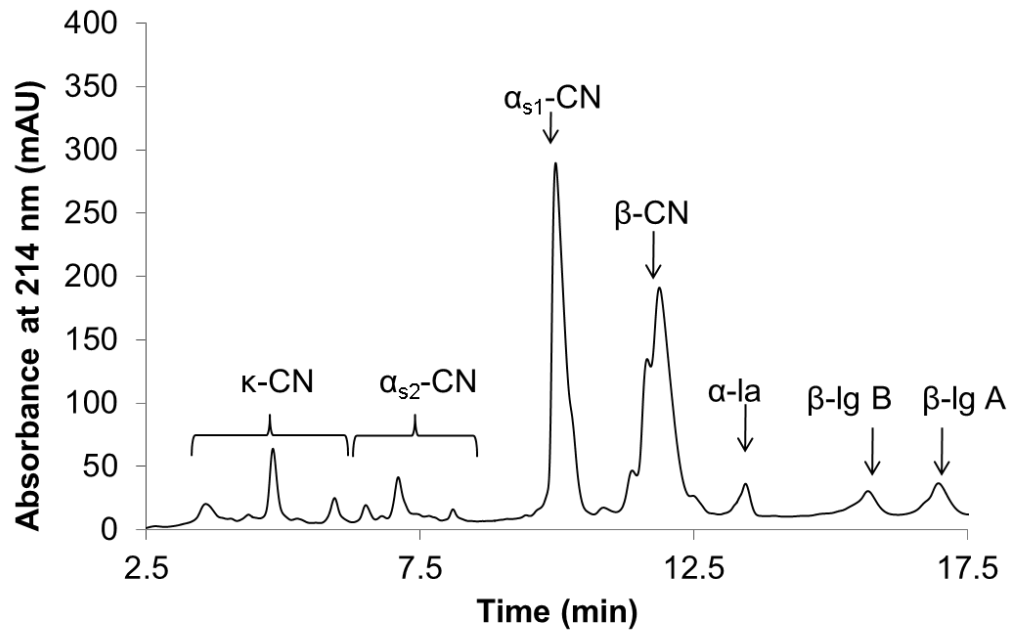
| Time (min) | % A | % B |
|------------|-----|-----|
| 0 | 80 | 20 |
| 3 | 80 | 20 |
| 13 | 60 | 40 |
| 33 | 40 | 60 |
| 35 | 0 | 100 |
| 40 | 0 | 100 |
| 40.5 | 80 | 20 |
| 45 | 80 | 20 |

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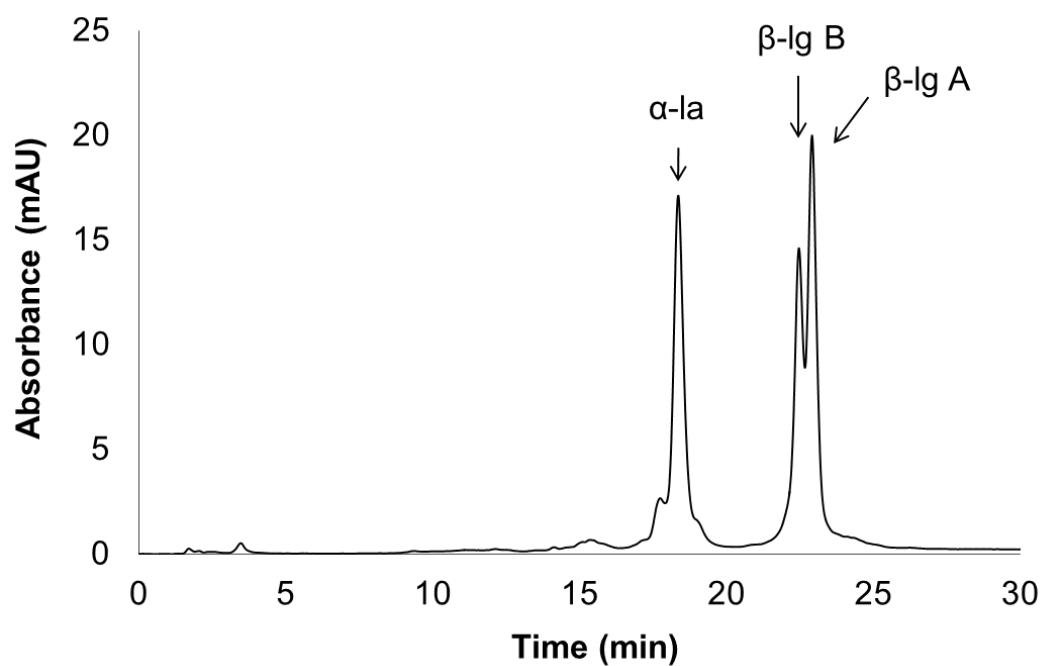
385 **Fig. 1**

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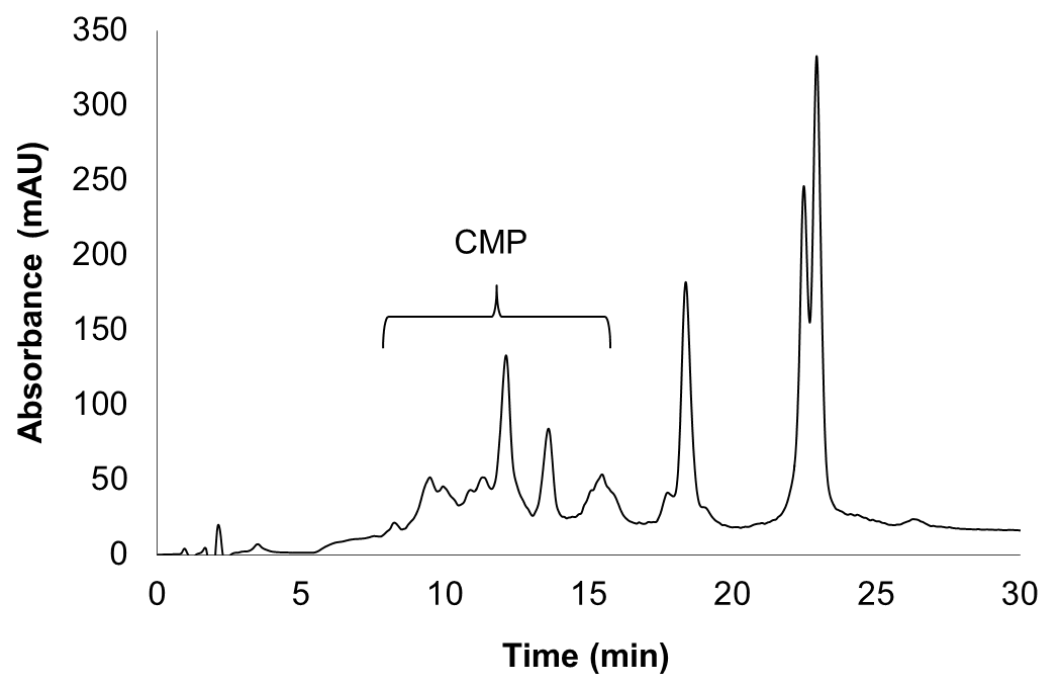
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387 (A)



388

389 (B)



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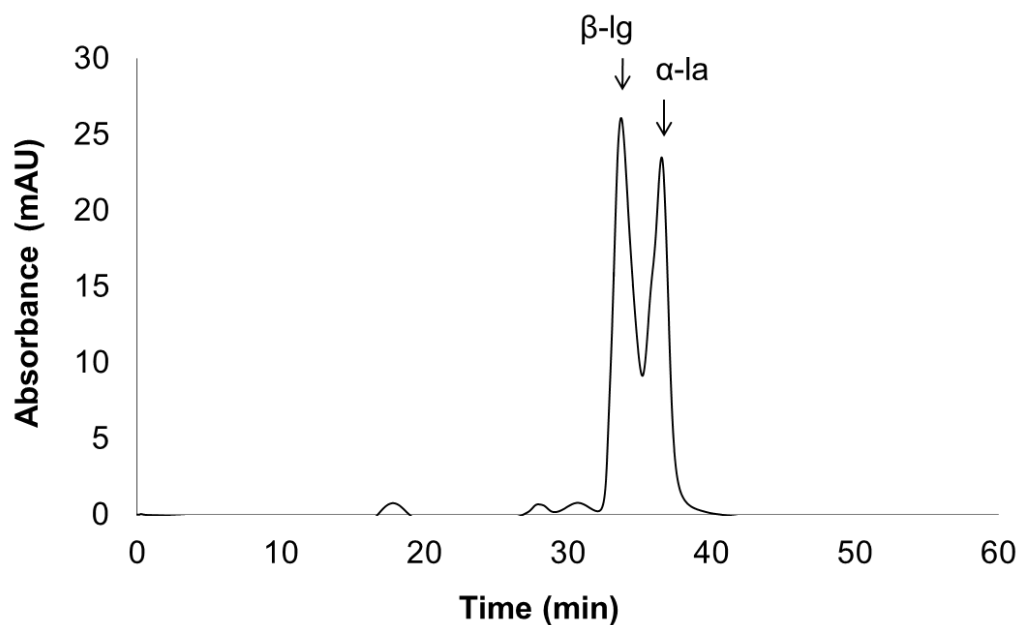
391 **Fig. 2**

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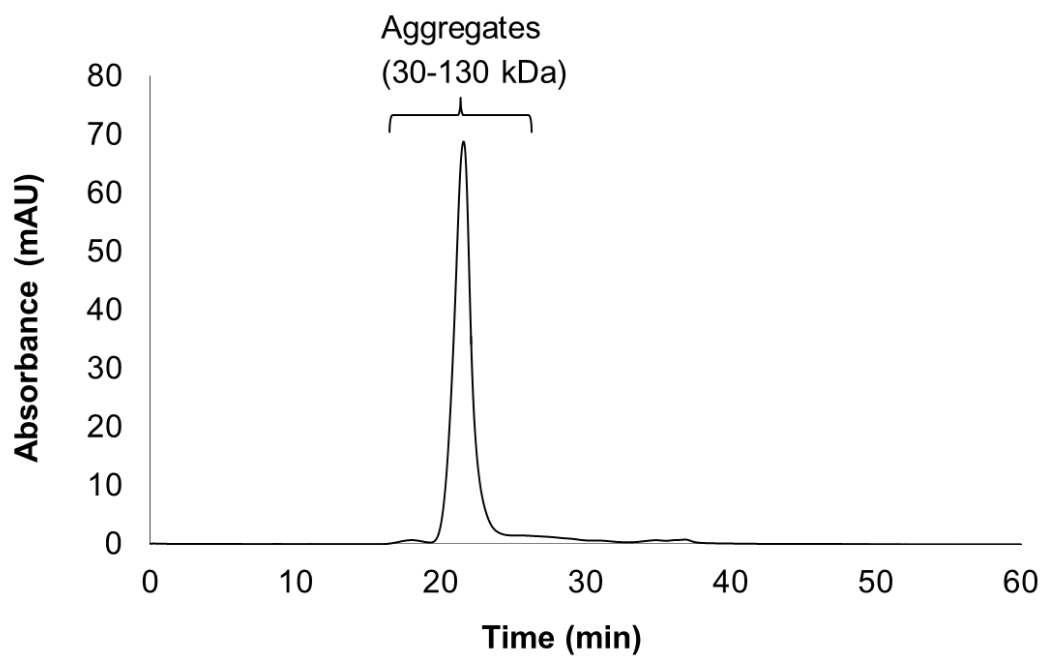
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393 (A)



394

395 (B)



396

397 **Fig. 3**

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