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A. Paul, C. Gaiani, L. Cvetkovska, C. Paris, M. Alexander, C. Ray, Gregory Francius, Sofiane El-Kirat-Chatel, Jennifer Burgain

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1 Deciphering the impact of whey protein powder storage on protein state and powder stability

2

3 A. PAUL¹, C. GAIANI^{1,2}, L. CVETKOVSKA¹, C. PARIS¹, M. ALEXANDER³, C. RAY³, G. FRANCIUS⁴, S.

4 EL-KIRAT-CHATEL⁴ & J. BURGAIN^{1*}

5

6 ¹ Université de Lorraine, LIBio, F-54000, Nancy, France

7 ² Institut Universitaire de France (IUF)

8 ³ Arla Foods Ingredients, Sønderupvej 26, 6920 Videbæk, Denmark

9 ⁴ Université de Lorraine, CNRS, LCPME, F-54000 Nancy, France

10

11 *Corresponding author: jennifer.burgain@univ-lorraine.fr

12

13 **Abstract**

14 Whey protein concentrate powders can be strongly affected by storage conditions inducing

15 both chemical and structural protein modifications. In the present work, a combination of

16 different storage conditions were evaluated, leading to the evaluation of 6 different batches

17 that were chosen in order to evaluate the impact of the following parameters: storage

18 temperature, storage duration and storage heat load (°C.days). In each set the relative humidity

19 was maintained at 70%. To estimate the impact of the three parameters, whey protein

20 lactosylation, denaturation and aggregation were followed. It was shown that the highest

21 temperatures (60 vs 55.5 °C and 41 vs 36 °C) led to greater protein modification, whereas the

22 duration (17 vs 14 days and 33 vs 30 days) and the total heat load (low vs high C°.days) did not

23 affect protein modification. Lactose content is known to be an important parameter for powder

24 browning. Here, we show that if lactose content is crucial for the level of initial process induced

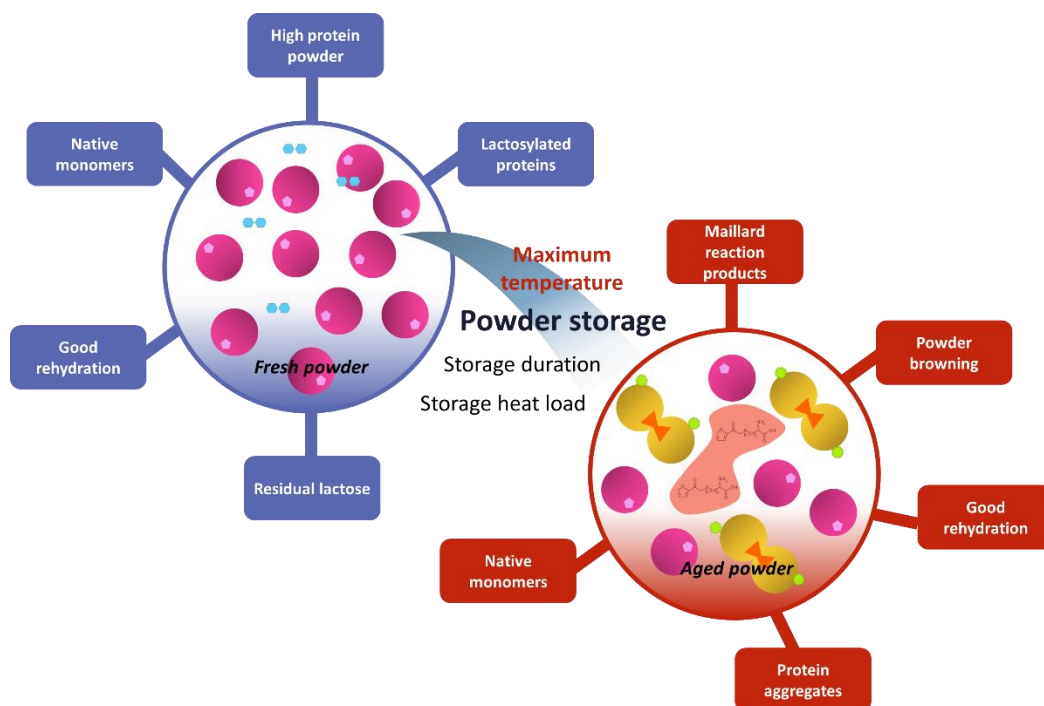
25 browning of the powder, the additional browning that occurs during storage is mostly
26 independent of lactose content. In addition, during storage, detected lactosylated proteins
27 were found to be inversely proportional to powder browning indicating the occurrence of
28 advanced Maillard reaction steps (e.g. dicarbonyl and AGEs formation and production of
29 melanoidins). Protein aggregates contained both β -lactoglobulin and α -lactalbumin and were
30 mostly formed by non-disulphide covalent bonds. Interestingly, despite protein structure being
31 strongly impacted, good powder rehydration properties were obtained for the studied
32 conditions.

33

34 **Key words:** dairy powders, whey proteins, β -Lactoglobulin, lactosylation, aggregation, Maillard
35 reaction, storage

36

37 **Graphical abstract**



38

39

40 1. Introduction

41 Dairy powders are considered stable products, at least within the limits of the best-before date.
42 In the past 50 years, with the emergence of filtration technology (*i.e.* microfiltration,
43 ultrafiltration, nanofiltration and reverse osmosis), new food ingredients were developed after
44 protein extraction and purification (*e.g.* micellar caseins, whey protein isolates) (Kelly, 2019;
45 Price, 2019). Their ability to be stored depends heavily on their intrinsic characteristics,
46 including composition and physicochemical properties, as well as the environmental conditions
47 (temperature, relative humidity) to which they are subjected during storage. However, several
48 studies evidenced that storage conditions can affect the structural and functional properties of
49 high protein dairy powders (Anema et al., 2006; Gazi & Huppertz, 2015). In this regard, a major
50 technological challenge is to limit the undesirable changes that occur when dairy powders are
51 stored in inadequate conditions (or suffer storage accidents), which lead to the degradation of
52 their techno-functional, sensory and/or nutritional properties. Most changes in dairy products
53 are temperature-dependent. For this reason, it is best practice to conduct storage trials at
54 different temperatures. These temperatures are often described as reflecting ideal, realistic,
55 and worst-case supply-chains (Andrewes, 2022). The time-dependence of changes is also a
56 critical point as the product shelf life can change with transport duration under varying
57 temperature conditions and depending on food temperature history. Modelling the time and
58 temperature dependence of reactions that occur in food, *i.e.* kinetic modelling, has been widely
59 discussed by (Capuano et al., 2018; M. A. J. S. Van Boekel, 2008; van Boekel, 2020).

60 A range of chemical reactions can lead to protein modification in dairy powders, in particular
61 when they are subjected to elevated temperatures and humidities during product shipment
62 and/or storage. One of the most important storage-induced change is the Maillard reaction
63 (MR), also known as non-enzymatic browning, initiated by the reaction of amino groups either

64 on proteins or peptides, with reducing sugars. In the context of dairy products, lysine residues
65 interact with lactose (Guyomarc'h et al., 2000), and lead to direct consequences on the
66 bioavailability of this essential amino acid (Hodge, 1953; Mehta & Deeth, 2016). The
67 lactosylation (reaction of lactose bonding to lysine side chain) rate is increased with increasing
68 temperature and relative humidity. At the intermediate and the final stages, sensory changes
69 occur as a result of the formation of volatile compounds and pigments. At the final stages,
70 brown pigments are responsible for the observed color changes, and represent a complex
71 mixture of polymers and co-polymers called melanoidins.

72 In dairy products, MR is influenced by many factors: temperature, storage duration, initial pH,
73 water activity (a_w), physical state and microstructure of the matrix and lactose concentration
74 (Baisier & Labuza, 1992; Mimouni et al., 2010). As an example, temperature has a crucial
75 importance considering that the Q_{10} (a factor related to the activation energy required for
76 reaction, equivalent to the fold change for every 10 °C) of the reaction is about 3-4, indicating
77 that reactions rate triples or quadruples every 10 °C increase in temperature (Boekel, 2001).

78 When trying to optimise storage conditions or to evaluate how variations during powder
79 shipment can impact powder functional properties, carefully selected chemical markers are of
80 fundamental importance. Regarding the MR, both the formation of intermediate products and
81 the disappearance of initial molecules have often been used as indicators of the different MR
82 stages (Ferrer et al., 2003). Different markers might be more suitable to use at each stage
83 (Leclère & Birlouez-Aragon, 2001; Leiva et al., 2017; Morales & van Boekel, 1998) such as
84 Amadori products, α -dicarbonyls, furosine, hydroxymethylfurfural, carboxymethyllysine, and
85 melanoidins.

86 In addition to chemical reactions attributed to MR, aggregation is another route to protein
87 modification (Liu & Zhong, 2013). Aggregation kinetics are extremely important as they can

88 impact the texture of dairy matrices (*e.g.* rheological features) and consequently their
89 functional and nutritional qualities (Nicolai et al., 2011). It has been shown that high
90 temperature storage (≥ 40 °C) leads to extensive protein lactosylation (Guyomarc'h et al., 2000),
91 and protein denaturation (Havea, 2006). Lactosylation is the first event occurring during high
92 temperature storage (Norwood et al., 2016). These lactosylated proteins are consumed by MR
93 and result in a decrease of lactosylated proteins detected during storage (Guyomarc'h et al.,
94 2000; Norwood et al., 2016). On the other hand, lactosylation gives strong reactive species that
95 promote protein aggregation (Norwood et al., 2016). Reactive amino acid residues such as
96 lysine could form intra- or inter-molecular cross-links leading to a loss in powder solubility of
97 milk protein concentrate powders during storage (Le et al., 2011, 2013). In addition, MR that
98 occur during powder shipment and/or storage, can lead to powder browning and correspond
99 to an irreversible stage.

100 This project aimed to better understand the impact of three storage parameters (storage
101 temperature, total heat load and storage duration) on the biochemical alterations of proteins
102 and their interactions. We illustrate that powder browning fold change and protein
103 modification (lactosylation, aggregation) that occur during storage is partially independent of
104 lactose content, but driven by the initial lactosylation level of whey proteins.

105

106 2. Material and methods

107 2.1. Dairy powders production

108 Dairy powders were produced and provided by Arla Foods Ingredients (AFI, Videbæk,
109 Denmark). A whey protein concentrate was obtained by ultra-filtration followed by reverse
110 osmosis. The concentrate was then spray-dried and the powders were directly conditioned into
111 aluminium bags (impenetrable for oxygen or water but without any flushed or gas inclusion)

112 and sealed. Three industrial powders were studied. Powder 1 and 2 originate from independent
113 concentrates, these later are produced independently and are considered as replicates despite
114 some slight differences on composition as illustrated Table 1. Powder 3 is a reference powder
115 mainly composed of β -Lactoglobulin (β -LG).

116

117 2.2 Powder storage conditions

118 After production, powders were immediately analysed for the identification of biochemical
119 properties of the fresh powders. Immediately afterwards, powders were stored in a climatic
120 chamber with controlled temperature and relative humidity (Climacell, MMM Medcenter,
121 Fisher Bio block Scientific, Illkirch, France). A schematic representation of the applied conditions
122 is shown in **Figure 1** and details are provided in **Table S1**. Each set corresponds to a storage path
123 with a temperature increase step (duration of 0.5 or 2 days) followed by storage at constant
124 temperature and finally a decrease step for a return to initial temperature conditions. During
125 the storage at constant temperature, the relative humidity was maintained at 70% for each set.
126 The total storage duration was comprised between 14 and 33 days. The heat load is
127 represented by the area under the curve (AUC). A low AUC (around 480 °C.days) was
128 established for sets 1, 2, 4 and 5. A high AUC (around 530 °C.days) was established for sets 3
129 and 6 meaning that these two conditions (sets 3 and 6) are comparable in terms of total heat
130 load.

131

132 2.3 Powder composition

133 2.3.1 Water content

134 Water content was determined by the loss of water mass of 1 g powder after drying during at
135 least 5 h at 103 °C until a constant dry powder mass was obtained following ISO 5537: 2004
136 (AFNOR, 2004).

137

138 **2.3.2 Proteins**

139 Nitrogen content of the powders was determined as previously described (Schuck et al., 2012).
140 The total nitrogen (TN) content, the non-casein nitrogen (NCN) content (soluble fraction at pH
141 4.6), and the non-protein nitrogen (NPN) content (soluble fraction after protein precipitation)
142 were determined by the Kjeldahl method. Nitrogen contents were converted into protein
143 contents using 6.38 as multiplying factor (Maubois & Lorient, 2016; *NF EN ISO 8968-1*, 2014).

144

145 **2.3.3 Lipids**

146 Total fat extraction was performed with a method adapted from (Folch et al., 1957). Powder (5
147 g) was dissolved into a mix of chloroform/methanol (2:1 (v/v)). The mix was stirred at 700 rpm
148 during 15 min. Then, the solvent was separated by filtration on a sintered glass filter (10 – 16
149 µm porosity) and recovered in a round-bottom boiling flask that was previously dried and
150 weighted. The solvent containing the total fat was removed with a rotary vacuum evaporator
151 (Heidolph laborator 4000) at 40 °C and 40 rpm. The boiling flask without solvent was dried in
152 an oven to 103 °C for 10 min to ensure complete evaporation of the solvent and finally
153 weighted.

154

155 **2.3.4 Minerals**

156 Mineral content was performed by mineralising 2 g of sample, and drying at 550 °C during 12
157 h (Mortensen & Wallin, 1989).

158

159 2.4 Chemical analysis

160 2.4.1 Identification and quantification of native proteins (by RP-HPLC)

161 Measurement and monitoring of protein denaturation was done with a reverse-phase high-
162 performance liquid chromatography (RP-HPLC) system adapted from (Kern et al., 2020). The
163 HPLC system (Shimadzu Corporation, Kyoto, Japan) was composed of a DGU-20A3 prominence
164 3 ways-degasser, SCL-10A VP system controller, a LC20-AD pump, a SIL-10AD VP auto-injector
165 and a SPD-M10A VP UV/VIS diode array detector, a regulator and column oven (Croco-cil 100-
166 040-220P, Cluzeau Info Labo). The data were processed on Shimadzu LC solution software. The
167 protein separation was performed using an Agilent (Santa Clara, USA) ZORBAX 300 Extend
168 column (250 mm length x 4.6 mm diameter, C18-bonded silica, 5 μ m bead size, 30 nm pore
169 size).

170 The sample was rehydrated at 3% (m/v) overnight at 4 °C in ultra-pure water. Protein
171 precipitation was performed by acidification with 1M acetic acid at pH 4.6 (solubility criteria).
172 A centrifugation at 14500 rpm during 25 min was then performed.

173 Two mobile phases were employed: a polar phase (A, ultra-pure water, acetonitrile and
174 trifluoroacetic acid at 89.9%, 10% and 0.1% v/v, respectively), a non-polar phase (B, ultra-pure
175 water, acetonitrile and trifluoroacetic acid at 10%, 89.9%, 0.1% v/v, respectively).

176 The mobile phase flow rate was kept at 1 mL.min⁻¹ and the temperature was set at 25 °C. The
177 pressure was 114 bars and 10 μ L of sample were injected per analysis. During analysis, the
178 gradient elution was carried out as follows: mobile phase was initially composed of 20% B-
179 phase and 80% A-phase. During the next first 35 minutes, B-phase was linearly increased from
180 20% to 65%. Then, it was raised up to 100% from 35 minutes to 45 minutes for finally being

181 decreased to 20% until 65 minutes. A typical RP-HPLC profile obtained with this method is
182 presented in **Figure S1**.

183 The peaks for each protein were identified using casein and whey protein standards (Sigma-
184 Aldrich). Any reduction in their area or any modification of their elution time were attributed
185 to non-native proteins. Non-native proteins include those that were denatured, aggregated or
186 lactosylated. The quantification was performed using standards curves (shown in **Figure S2**) for
187 β -LG (A and B variant), α -lactalbumin (α -LA), individual caseins (β -casein, α -casein); other
188 proteins (*i.e.* CMP, BSA and glycosylated protein) were only identified (using standards) but not
189 quantified.

190

191 2.4.2 Protein lactosylation (using UHPLC-MS)

192 The samples were rehydrated a day before at 5% (m/v) at 4 °C in ultra-pure water and acidified
193 with acetic acid (1 M) (255 μ l of acid with 1.7 mL of sample). The solutions were vortexed and
194 kept one hour at 4 °C. A centrifugation at 14500 rpm during 25 min was then performed. Finally,
195 the sample was filtrated (0.2 μ m) and diluted (1/10) with ultra-pure water.

196 Qualitative and semi-quantitative analysis of the different lactosylated forms of protein was
197 realized on a UHPLC-MS system (ThermoFisher Scientific, San Jose, CA, USA) consisting of a
198 quaternary U3000 solvent delivery pump connected to a photodiode array detector (PDA) and
199 a LTQ^{XL} mass spectrometer equipped with an atmospheric pressure ionization interface
200 operating in electrospray positive mode (ESI⁺). Ten microliters of sample were separated on a
201 C18 Alltima reverse phase column (150 x 2.1mm, 5 μ m – Grace/Alltech, Darmstadt, Germany)
202 equipped with a C18 Alltima pre-column (7.5 x 2.1mm, 5 μ m) at 25 °C. The flow rate was set at
203 0.2 mL.min⁻¹ and mobile phases consisted of water modified with trifluoroacetic acid (0.1%) for
204 A and acetonitrile modified with trifluoroacetic acid (0.1%) for B. Proteins were eluted using a

205 linear gradient from 10% to 98% of B for 15 min. Mass spectrometric conditions were as
206 follows: spray voltage was set at + 5 kV; source gases were set (in arbitrary units.min⁻¹) for
207 sheath gas, auxiliary gas and sweep gas at 20, 5 and 5, respectively; capillary temperature was
208 set at 350 °C; capillary voltage at + 26 V; tube lens, split lens and front lens voltages at + 130 V,
209 - 42 V and - 5.75 V, respectively.

210 Ion optics parameters were optimized by automatic tuning using a standard solution of BSA at
211 0.1 g.L⁻¹ infused in mobile phase (A/B: 50/50) at a flow rate of 5 µL.min⁻¹. Semi-quantitative
212 screening of total soluble proteins was realized using UV230 absorbance. Structural elucidation
213 of the different lactosylated forms of protein was realized thanks to full scan MS spectra
214 performed on LTQ^{XL} analyzer from 1500 to 4000 m/z, and subsequent assignment of charge
215 states of proteins.

216 In addition, semi-quantitative analysis of lactosylated forms was achieved through specific mass
217 spectrometry screening. Raw data were processed using the XCALIBUR software program
218 (version 2.1, <http://www.thermoscientific.com>).

219

220 **2.4.3 Protein aggregation (using size exclusion chromatography)**

221 The samples were rehydrated a day before at 1% (m/v) at 4 °C in ultra-pure water. A
222 centrifugation 2 min at 14500 rpm was followed by 0,2 µm filtration. For HPLC-SEC analyses
223 (Shimadzu Corporation, Kyoto, Japan, see section 2.4.1 for more details), 80 µg of protein are
224 injected in a series of Agilent Bio SEC-5, 150 Å, 7.8 x 300 mm, 5 µm column, followed by Agilent
225 Bio SEC-5, 400 Å, 7.8 x 300 mm, 5 µm preequilibrated in PBS 1X pH 6.8. Each run is performed
226 at 25°C at 1mL/min during 30 minutes and all samples are performed in triplicates. Data and
227 pics integration are obtained at 280 nm with the Shimadzu LC solution software. For low
228 pressure chromatography, a HiPrep™ 16/60 Sephacryl® S-200 HR (Cytiva) was connected to an

229 ÄKTA start liquid chromatography system (GE Healthcare), and preequilibrated in PBS 1X pH
230 6.8. Runs are performed at 1 mL.min⁻¹ during 120 min, and proteins are fractionated each 5 mL
231 using a fraction collector. Pics integrations and measurements are performed at 280 nm with
232 Unicorn™ Start 1.0 (GE Healthcare). Proteins in fractions are precipitated with 4 volumes of
233 cold acetone before loading on 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Biorad) in
234 presence of DTT in samples. After 120V running, SDS-PAGE gels are colored with InstantBlue®
235 Coomassie Protein Stain (Abcam).

236

237 **2.5 Functional properties**

238 Rehydration ability of spray-dried powders was evaluated through wettability, dispersibility and
239 solubility indices. The IDF standards were adapted (IDF 87: 1979 for wettability and
240 dispersibility (IDF, 1979) and ISO 8156: 2005 for solubility (IDF, 2005)).

241

242 **2.5.1 Wettability**

243 Wettability was evaluated by the time in seconds taken by 13.0 ± 0.1 g of powder to become
244 wet when gently deposited at the surface of 125.0 g ± 0.1 g distilled water in a 250 mL beaker
245 following IDF 87: 1979.

246

247 **2.5.2 Dispersibility**

248 Dispersibility index was measured by a method adapted from IDF 87: 1979. 13.0 ± 0.1 g powder
249 was gently deposited on the surface of 125 g water at 20 °C placed in a 400 mL beaker. The
250 mixture was manually stirred for 20 s, then the mixture was allowed to rest for 30 s. After that,
251 the mixture was passed through a 120 µm sieve. The total dry matter of the liquid was
252 determined by drying at 103 °C for 5 h.

$$\text{Dispersibility (\%)} = \frac{W \times T}{100 - (E + T)} \times \frac{100}{S}$$

253

254 With:

255 - W: distilled water mass (g);

256 - T: dry matter content (% w/w) of the liquid;

257 - E: dry matter content (% w/w) of the sample;

258 - S: sample mass (g).

259

260 2.5.3 Solubility

261 Solubility was characterized as the percentage of powder able to be dissolved in distilled water.

262 It was determined by the addition of 2.5 ± 0.1 g powder in 17.5 mL distilled water in a 50 mL

263 centrifugation tube followed by 30 s stirring and centrifugation at $2\ 294 \times g$ during 10 min. Then,

264 the supernatant was removed and the pellet was added to 20 mL distilled water, followed by

265 stirring and centrifugation at $2\ 294 \times g$ during 10 min. The pellet was desiccated at $103\ ^\circ\text{C}$

266 overnight to determine the mass of insoluble residues, which allows determining the solubility

267 index adapted from ISO 8156: 2005.

$$\text{Solubility (\%)} = 100 - \frac{M \times 100}{S}$$

268

269 With:

270 - M: sediment mass after desiccation (g);

271 - S: sample mass (g).

272

273 **2.5.4 Size of aggregates**

274 The particle size after powder reconstitution at 5% (m/m) was followed using the granulometer
275 Mastersizer 3000 (Malvern Instruments, UK). The granulometer Mastersizer 3000 was
276 equipped with the Hydro MV unit. The sample is passed through the measuring cell using a
277 centrifugal pump. The particle size is determined by a sequential combination of measurements
278 with red and blue light sources. For one measurement, the integration time of each light source
279 was of 10 s. Three repetitions were performed for each sample.

280

281 **2.5.5 Color measurements**

282 Powder color was determined using a CR-400 chromameter (Konica Minolta). For each sample,
283 10 g of powder was poured in a petri dish and color measurement was performed according to
284 the three-color coordinates (L^* , a^* , b^*). The L^* value represents sample luminosity, varying
285 from black (0) to white (100), the a^* value represents the color varying from red (+) to green (-
286) and the b^* value represents the color varying from yellow (+) to blue (-). Browning index (BI),
287 defined as brown color purity, is one of the most common indicators of browning in food
288 products (Norwood et al., 2017).

$$BI = 100 * \frac{(x - 0.31)}{0.17}$$

289

290 With $x = \frac{(a^* + 1.75 L^*)}{(5.645 L^* + a^* - 3.012 b^*)}$

291 Each measured value was the mean of 3 independent measurements per powder and per set.

292

293 **2.7. Statistical analysis**

294 Two whey protein concentrates presenting the same formulation were spray-dried
295 independently. For each powder, 3 experimental measurements were performed. The
296 repeatability can thus be estimated by the three measurements performed on a same sample
297 and reproducibility is evaluated through the comparison of results between independent
298 powders. Data were reported as mean values plus standard error of the mean of a least three
299 replicates. The significance level using a paired two-tailed Student's t-test was set to P values
300 N.S. (non-significant) >0.05, *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.

301

302 3. Results

303 3.1. Evolution of powder functional properties during storage

304 3.1.1. Powder composition

305 The chemical composition of fresh powders was evaluated (**Table 1**). It can first be observed
306 that, as expected, the composition for powders 1 and 2 are very similar but not identical.
307 Proteins represent the major constituents of the powders, with 80 - 84% content (by mass).
308 Residual lipids were found for powders 1 and 2 and lactose content was 1.5%. The main
309 difference between the powders is the amount of in residual caseins, which represent 0.9% of
310 total proteins in powder 1 but 5.4% of total proteins in powder 2. The reference powder differs
311 according to the absence of lipids and lactose. β-LG represent the major protein with no
312 detectable residual individual caseins and α-LA.

313

314 Lipids were analysed during storage and no significant differences were observed between
315 fresh and aged powders. The total lipid percentage remained the same as well as the
316 saturated/monosaturated/polyunsaturated fractions, meaning that no lipid oxidation

317 occurred. This can be expected given the fact that fatty acids in milk are mainly saturated (data
318 not shown).

319

320 **3.1.2. Powder color**

321 During storage, the powder appearance changed with the development of a brown color. The
322 MR is known to be one of the major deteriorative reactions during food storage. It impacts the
323 powder appearance leading to a brown color due to the production of melanoidins (Lin et al.,
324 2022). An easy way to evaluate the MR development is by measuring color parameters, making
325 it possible to calculate the BI (**Figure 2-A**). Images of powders spread in Petri dishes before and
326 after storage were taken for each set (**Figure 2-B**). From these images and BI values, it is clearly
327 visible that sets 1, 2 and 3 present a more pronounced color evolution than sets 4, 5 and 6,
328 suggesting that high temperature storage has a much stronger impact on browning than either
329 storage duration or total heat load. Interestingly, if the reference powder (where no lactose is
330 detectable) shows a very light color, storage induces the same increase in browning as for
331 powder 1 & 2 (fold x3-4 for sets 1, 2 & 3, and fold x2 for sets 4, 5 & 6; **Figure 2-C**). These
332 observations suggest that although lactose content is crucial for the initial browning value of a
333 powder (Nasser et al., 2017; Norwood et al., 2017) the browning that occurs during storage of
334 these powders is independent of lactose content. Significant differences were observed
335 between sets 1 and 2/3; the BI being more pronounced after storage at 60 °C than 55.5 °C. The
336 highest BI was observed for a residence time at the highest temperature meaning that
337 temperature triggers the browning reaction more than the total heat load (also defined here
338 as the AUC) or the duration of storage.

339

340 **3.1.3. Powder rehydration**

341 It is commonly believed that the rehydration process consists of three sequential stages, which
342 are wetting, dispersion and solubilisation (Crowley et al., 2016). Wetting is the first step as the
343 particles come in contact with the liquid, while dispersion and solubilisation are the critical
344 phases, when primary particles start to release material from the particle surface into the liquid
345 (Forny et al., 2011; Ji et al., 2016). The evaluation of powder functional properties to describe
346 rehydration ability was done with IDF standards. The results for solubility and dispersibility are
347 presented in **Table S2**. All the powders in the present study were considered non-wettable (time
348 > 1800 s). It is well known that whey protein powders show poor wetting behaviour, the
349 material floats on the water surface, which is considered to be the rate-limiting factor
350 particularly for whey proteins (Gaiani et al., 2009).

351 On the other hand, it was observed that whatever the storage condition, the powders
352 presented excellent rehydration capacity with high dispersibility. However, it can be noticed
353 that a small decrease in solubility can be observed for the powders stored in the conditions of
354 set 1, corresponding to the highest temperature.

355

356 **3.2. Protein modification during powder storage**

357 **3.2.1. Protein lactosylation and aggregation**

358 Native proteins were quantified by RP-HPLC (**Figure 3-A**). The mean values were calculated from
359 data obtained for powders 1 and 2. It can be seen that the differences between powder 1 & 2
360 are very low, meaning that powders coming from two different concentrates show very similar
361 evolution of native proteins during powder storage. The lowest content of native proteins was
362 observed for powders stored according to conditions in sets 1, 2 and 3. This means that the
363 percentage of native proteins is strongly linked to the maximum storage temperature and not
364 to the duration (same observation as the BI evolution). Even if the proportion of native proteins

365 in the reference powder was more important than in powders 1 and 2, these powders were
366 also strongly affected by storage conditions.

367 Non-native proteins are represented by lactosylated and/or denatured and/or aggregated
368 proteins. β -lactoglobulin lactosylation was followed as a function of powder storage conditions.

369 For powders 1 and 2, it can first be observed that the lactosylation was more pronounced for
370 sets 4, 5, 6 than for sets 1, 2, 3 (**Figure 3-B**). If we now consider, in detail, the number of lactose
371 linked per protein, it appears that sets 4, 5 and 6 have a higher number of grafted lactose
372 molecules per protein than sets 1, 2 and 3 (**Figure 3-C**, mean of powders 1 & 2). Nevertheless,
373 the number of lactose molecules detected per β -LG (maximum detected = 4 lactose / β -LG) is
374 poor considering that up to eleven lactose could theoretically be bound to β -lactoglobulin
375 (Gasparini et al., 2020; Marvin et al., 2002; Morgan et al., 1998, 1999). As shown by the
376 previously shown browning data (**Figure 2**), the MR evolved faster for sets 1, 2 and 3. They were
377 stored at higher temperatures and the lactosylated proteins are no longer detectable as they
378 are engaged in advanced MR steps (*e.g.* for the production of brown pigments; (Boekel, 2001;
379 Guyomarc'h et al., 2000). Regarding the reference powder, the initial lactosylation level (*i.e.*
380 lactosylated proteins in the fresh powder) was around 3% meaning that the successive
381 industrial processes applied to obtain the concentrate and the powder (spray-drying step) lead
382 to protein lactosylation. In this case, even if no residual lactose can be detected in the powder,
383 the 3% of already-lactosylated proteins, when stored at elevated temperature and relative
384 humidity, can continue their chemical evolution due to MR (Norwood et al., 2017). As such,
385 during storage, no additional lactosylation occurred in the reference powder (no lactose in this
386 powder; **Figure 3-B**) even though browning increased due to MR (**Figure 2-C**). When no
387 lactosylation occurs (as in the reference powder), a lower level of lactosylated protein is
388 detected with high temperature storage (see **Figure 3-B** set 1, 2 & 3 vs. fresh reference powder).

389 As in the case of powder 1 and 2, the protein lactosylation for the reference powder is the
390 lowest for set 1, reinforcing the idea that lactosylated proteins are consumed by MR during
391 high temperature storage (Guyomarc'h et al., 2000).
392 Lactosylated proteins are more prone to aggregation (Norwood et al., 2016, 2017). This can be
393 confirmed by a higher proportion of large insoluble protein aggregates and precipitates for sets
394 1, 2 and 3 as shown after rehydration (**Figure 4**). It can be seen, for the first time, a different
395 behaviour between powders 1 and 2. In fact, though the chemical characterization of the two
396 powders was similar, powder 2 presents a more significant proportion of very large aggregates
397 and precipitates when compared to powder 1. It can be hypothesised that the presence of local
398 physicochemical variations triggers changes favouring protein aggregation. At the same time,
399 powder 2 shows a higher number of individual caseins in bulk, meaning that aggregation
400 between whey proteins and residual caseins could also be possible.

401

402 3.2.2. Identification of proteins present in aggregates

403 In order to further analyse the composition of protein aggregates, proteins in powder 1 & 2
404 were separated with SEC-HPLC. As shown in **Figure 5**, β -lactoglobulin and α -lactalbumin are
405 both subjected to aggregation, shown by the decreased level of native proteins' pic(peak) area.
406 As also shown in **Figure 3-A**, the lowest level of native proteins is observed for set 1, 2 & 3. This
407 decreased level of native proteins is correlated with an increased level of protein complexes of
408 higher molecular weight, indicating large accumulation of aggregates. In order to investigate in
409 more detail which proteins take part in the formation of the soluble aggregates, proteins from
410 fresh and set 1 powder 1 were injected into a low-pressure chromatography system and
411 fractionated, collected, and loaded on an SDS-PAGE (**Figure 6-AB**). First observations reveal that
412 stored powder (SET 1) is composed of a very large amount of soluble aggregates that are

413 formed by non-disulphide covalent bonds (cloudy migration of proteins in **Figure 6-B**, (Morgan
414 et al., 1999; Norwood et al., 2017)). From a detailed inspection of the SDS-PAGE profiles, it
415 appears that soluble aggregates are small (from 30 to 300kDa, *i.e.* approximately 2 to 20
416 proteins), and no degradation of protein occurs (no smear below β -lactoglobulin and α -
417 lactalbumin bands). As shown by the superposition of the 2 chromatograms in **Figure 6-C**, the
418 increased level of aggregates fits exactly with the decreased level of native β -lactoglobulin and
419 α -lactalbumin, indicating that aggregates are formed with both proteins.

420

421 **4. Discussion**

422 *Powder browning.* Interestingly, powders 1 and 2 appear very similar in terms of color evolution
423 (the values are superimposed) even if they originate from the drying of independent
424 concentrates. The behaviour of these two powders presenting the same composition, but
425 being industrially produced independently, mean that ageing process that the powders
426 underwent is relatively constant between productions. This is particularly important for
427 industry/dairy companies as when predicting the powder shelf life, they must ensure that the
428 proposed model perfectly fits the behaviour of all of their products.

429 The lactose content was already shown to have a significant effect on whey protein powders
430 ageing (Norwood et al., 2017). Crystallization of amorphous sugars has a significant effect on
431 the quality and shelf life of a number of products including high protein dairy powders. In
432 amorphous systems, crystallization occurs as a result of increased molecular mobility above the
433 glass transition. To discriminate the possible lactose crystallisation in the stored powders, X-ray
434 diffraction was performed (data not shown). The absence of characteristic peaks in XRD
435 patterns suggests an amorphous state of the molecules. This could be partly explained due to
436 the fact that storage conditions were below the glass transition temperature of lactose, and

437 partly due to the protecting role of whey proteins (Fan & Roos, 2015). Accordingly, lactose
438 content has a strong impact on powder browning considering browning difference between
439 the reference powder (no residual lactose detected) and powder 1 & 2 (1.5% lactose), even in
440 fresh powder. Nevertheless, despite the fact that no residual lactose can be detected in the
441 reference powder, the BI of this powder increased slightly during storage. It may be
442 hypothesised that due to the different processes applied to the β -LG concentrate, only 3% of
443 lactosylated proteins are sufficient to engage in MR during storage, leading to the production
444 of brown pigments, as well as for powders 1 and 2. Unexpectedly, the increase in browning in
445 the reference powder and powders 1 & 2 is similar (**Figure 2-C**), suggesting that in these
446 powders, the browning that occurs during storage does not depend on lactose content but
447 mostly on proteins in advanced-MR steps already present before storage. On the contrary, no
448 color evolution was quantified for lactose-reduced WPI powders (Norwood et al., 2017) or β -
449 LG powders (Thomas et al., 2005). As a consequence, it appears that the lactose content is a
450 very important parameter for fresh powder browning, as shown by the browning index of fresh
451 reference powder and fresh powder 1 & 2. Nevertheless, from the initial powder browning, our
452 results hypothesized that lactose is not involved in browning during storage. From the results
453 published by Norwood et al, 2017, a same tendency can be observed. In fact, the authors
454 compared levels of lactosylated β -LG during whey powders storage at 40 °C and 60 °C. They
455 evidenced a higher browning index for powders presenting a higher lactose content but it can
456 be also seen that color evolution is clearly linked to initial content in lactosylated β -LG
457 (Norwood et al., 2017). Consequently, the levels of lactosylated proteins in the fresh product
458 should be carefully monitored as they could evolve later into melanoidins during product
459 storage.

460

461 *Denaturation/lactosylation.* Theoretically, β -LG has the ability to bind more than 15 lactose
462 molecules per protein (Gasparini et al., 2020; Morgan et al., 1998). In the present study, LC/MS
463 analysis evidenced that a maximum of 4 lactose per protein were detected. The reason for this
464 result could first be assigned to restricted accessibility of amine groups to lactose molecules. In
465 fact, lactosylation of a folded protein can only be done on exposed amine groups and, if
466 additional heat treatment is performed, even in the dry state, a protein unfolding can occur
467 thereby exposing previously hidden amine groups susceptible to lactosylation. Secondly, it can
468 be hypothesised that if the MR is sufficiently rapid, lactosylated proteins are engaged in
469 advanced MR products resulting in a decrease of free lactose molecules able to interact with
470 amine groups. Several studies suggested a loss of organised tertiary protein structures upon
471 covalent binding of sugar molecules that induces a steric stress favouring protein unfolding by
472 a decrease in intra/inter molecular interactions (Hiller & Lorenzen, 2010; Wooster & Augustin,
473 2007). LC/MS data showed that set 1, 2 & 3 (which are more sensitive to browning) contained
474 less lactosylation than set 4, 5 & 6. This observation is in agreement with previous studies
475 showing that, when stored at high temperatures, lactosylated proteins present in powders are
476 no longer detectable as they are engaged in advanced MR steps (Guyomarc'h et al., 2000;
477 Norwood et al., 2016, 2017; M. Van Boekel, 1998). Interestingly, as the reference powder does
478 not contain lactose, no additional lactosylation occurred during storage. Indeed, the
479 lactosylation level of β -lactoglobulin decreased in set 1, 2 & 3 compared to fresh powder,
480 illustrating the Schiff base consumption by intermediate MR. Powders 1 & 2 show strong
481 protein lactosylation. Despite this observation, no additional browning could be linked to these
482 lactosylation profiles. As browning for the reference powder, and powders 1 & 2 are similar
483 during storage (**Figure 2-C**), and no lactosylation is detected on the reference powder (**Figure**

484 **3-B**), the extra-lactosylation occurring in powders 1 & 2 during storage could not be linked to
485 an increased in browning.

486

487 *Protein aggregation.* During powder storage, and especially for the sets 1, 2 and 3, large protein
488 aggregates and precipitates composed by β -lactoglobulin and α -lactalbumin were detected
489 after powder rehydration (**Figure 4, 5 & 6**). These insoluble aggregates are formed in the dry
490 state during storage and it can be observed in the present study. Even if the powders 1 and 2
491 are similar (except for individual caseins content), protein aggregation and precipitation is
492 significantly more pronounced for the second powder when compared to the first one. Whey
493 protein denaturation and/or aggregation was previously studied as a consequence of dry
494 heating (Guyomarc'h et al., 2015). Aggregates can result from covalent crosslinks, other than
495 disulphide bonds, between individual monomers (**Figure 6** and (Norwood et al., 2017)). Non-
496 disulphide covalent bonds aggregates comprised 2 to 20 proteins and no proteolysis occurred
497 during storage. Dehydroalanine (DHA), and DHA-derived lanthionine and lysinoalanine has
498 been detected in infant milk formula (Chen et al., 2019). These modified aliphatic amino acids
499 are subjected to form intra- and intermolecular crosslinks (Linetsky et al., 2004), as reported in
500 milk powder by Chen et al, and could be responsible of the identified crosslinks in **Figure 6** (Chen
501 et al., 2019). As shown by chromatograms superposition **Figure 6-C**, dead volume intensity is
502 not affected by storage. This observation confirms that aggregates are small and never exceed
503 a size of 250 kDa (limit of resolution of the column), and suggests that most of the aggregates
504 are exclusively formed with non-disulphide bonds. It has been shown before that the size of
505 protein aggregates depends on the physicochemical conditions of the medium and potentially
506 the protein ingredient history (Barroso da Silva et al., 2020). For example, storage temperature
507 and time (Norwood et al., 2017) but also a_w and pH of the powder (Gulzar et al., 2011)

508 dramatically affect the reaction rate and the nature of the end products formed. This means
509 that the presence of residual lactose in high protein dairy powders can strongly affect protein
510 functionality. In fact, (Zhou & Labuza, 2007) showed that the absence of lactose in a WPI
511 powder did not lead to greater structural changes compared to pure β -LG powder when stored
512 at 45 °C for 2 weeks. In our samples, the main difference between the chemical composition of
513 the two powders is the quantity of individual caseins, these differences may explain the
514 presence of larger insoluble aggregates in powder 2.

515

516 *Powder functional properties.* Regarding WPI powders, it has been demonstrated that the level
517 of lactosylated proteins increased from the first months of storage reaching a maximum, then
518 decreased with storage time (Norwood et al., 2016). The decrease in protein lactosylation was
519 concomitant with an increase in aggregated proteins and BI. The authors hypothesised that
520 proteins were first modified by lactosylation and then, protein unfolding as well as further
521 reaction of lactosylated lysines via the Amadori product contributed to the formation of
522 aggregated proteins and the browning of the powder. The impact of these structural changes
523 was observed in the heat-induced aggregation properties but not on the foaming properties
524 (Norwood et al., 2016). In the present work, the studied functional properties are linked to the
525 powder rehydration (wettability, solubility and dispersibility) which were not strongly affected.
526 Only a slight impact on powder solubility was observed which can be attributed to the
527 formation of insoluble protein aggregates.

528

529 5. Conclusion

530 During dairy powder storage, a range of chemical reactions can lead to protein modification, in
531 particular when they are subjected to elevated temperatures and humidities. One of the most

532 important reactions is the MR which is responsible for a loss of nutritional value and can
533 strongly impact powder functional properties. Protein lactosylation could lead to protein
534 denaturation and aggregation, and reciprocally, and are the different steps leading to the
535 formation of brown pigments (melanoidins) due to MR during powder storage. In the present
536 study, we showed that the presence of 1.5% lactose content (powders 1 and 2) was partly
537 responsible for protein lactosylation, as around 8% of proteins were already lactosylated in the
538 fresh powder. On the other hand, the reference powder, where no lactose traces could be
539 found, also showed a lactosylated proteins (albeit of around 3%). Interestingly, we show that,
540 during storage, browning is nearly independent of lactose content as the browning index
541 evolved similarly in these powders.

542 Regarding storage parameters, the maximum temperature appears to be of great importance
543 for all powders involved in this work. The state of the proteins strongly differs depending on
544 the storage history. Higher temperatures lead to complete MR until the production of brown
545 pigments (sets 1, 2 and 3). Powders stored at lower temperatures present a more important
546 percentage of proteins in the early MR steps, in particular as lactosylated proteins (sets 4, 5
547 and 6).

548

549 Currently, manufacturers implement processes such as ultrafiltration to produce their WPI
550 powders with limited residual lactose of around 2% (w/w) or less. Given the results presented
551 here, it is important to highlight that the limitation of residual lactose in the final product is
552 important, but so is the amount of lactosylated proteins, as they drive the subsequent browning
553 during storage. Once the MR is initiated, if the physicochemical and environmental conditions
554 are favourable, the reaction will continue until reaching the final steps, despite an absence of
555 residual lactose in the powder. In a future work, it would be interesting to determine how

556 lactosylated proteins will evolve if storage is carried out under more industrially realistic
557 conditions by reproducing temperature and relative humidity evolution during time based on
558 recorded data during industrial product shipment.

559

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565

566 7. Conflict of interest

567 MA and CR work for ARLA Foods Ingredients Group. No potential conflict of interest was
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569

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Table 1: Chemical composition (n=2) for powders 1, 2 and reference

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	Powder 1	Powder 2	Reference
Lipids (g.100g ⁻¹)	6.4 ± 0.4	4.8 ± 0.0	-
Lactose* (g.100g ⁻¹)	1.5	1.5	-
Ashes (g.100g ⁻¹)	2.8 ± 0.0	2.7 ± 0.1	2.3 ± 0.1
Water (g.100g ⁻¹)	5.0 ± 0.0	4.3 ± 0.1	4.6 ± 0.1
Total proteins (g.100g⁻¹)	79.6 ± 0.1	84.1 ± 1.1	83.9 ± 3.9
<i>Caseins (% of total proteins)</i>	0.9 ± 0.4	5.4 ± 0.3	-
<i>β-LG (A) (% of total proteins)</i>	26.0 ± 0.3	24.7 ± 0.2	31.6 ± 0.1
<i>β-LG (B) (% of total proteins)</i>	48.5 ± 0.2	46.5 ± 0.2	68.4 ± 0.4
<i>α-LA (% of total proteins)</i>	24.6 ± 0.2	23.4 ± 0.1	-

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*Data provided by AFI

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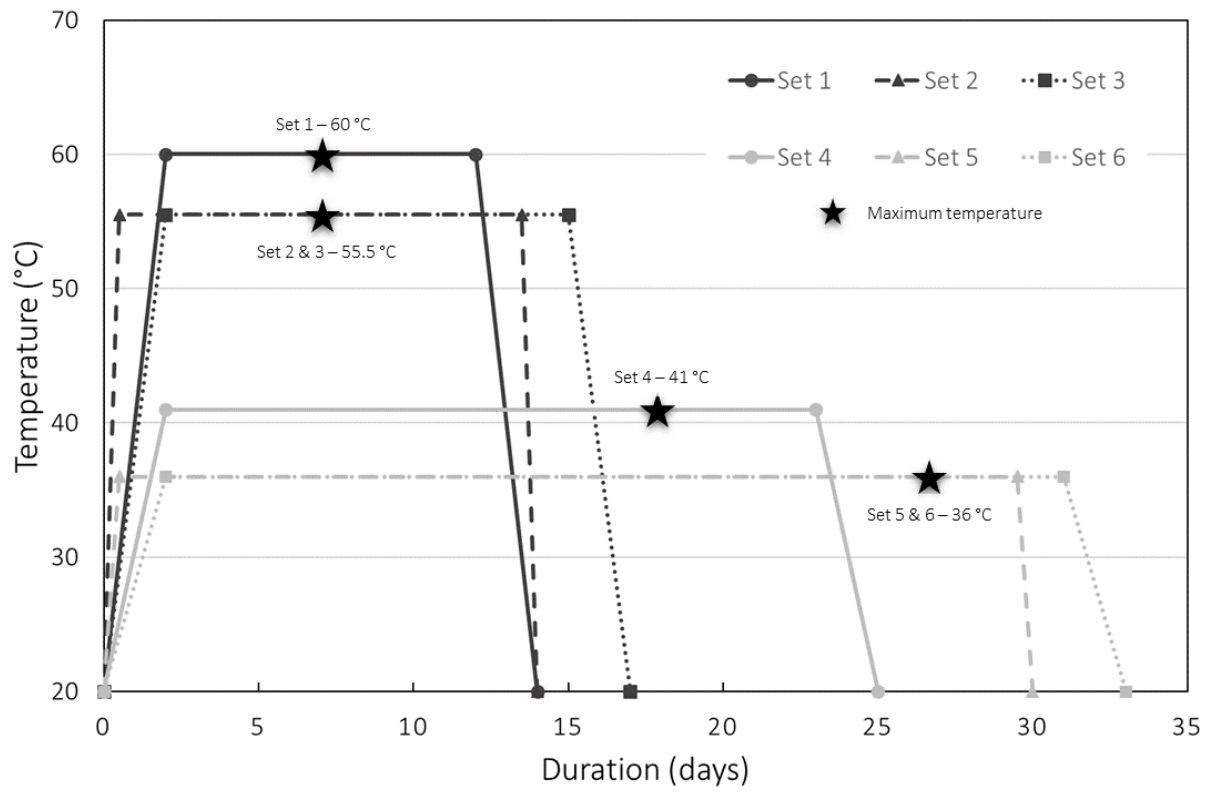


Figure 1: Schematic representation of storage path used and showing the temperature variation as a function of time

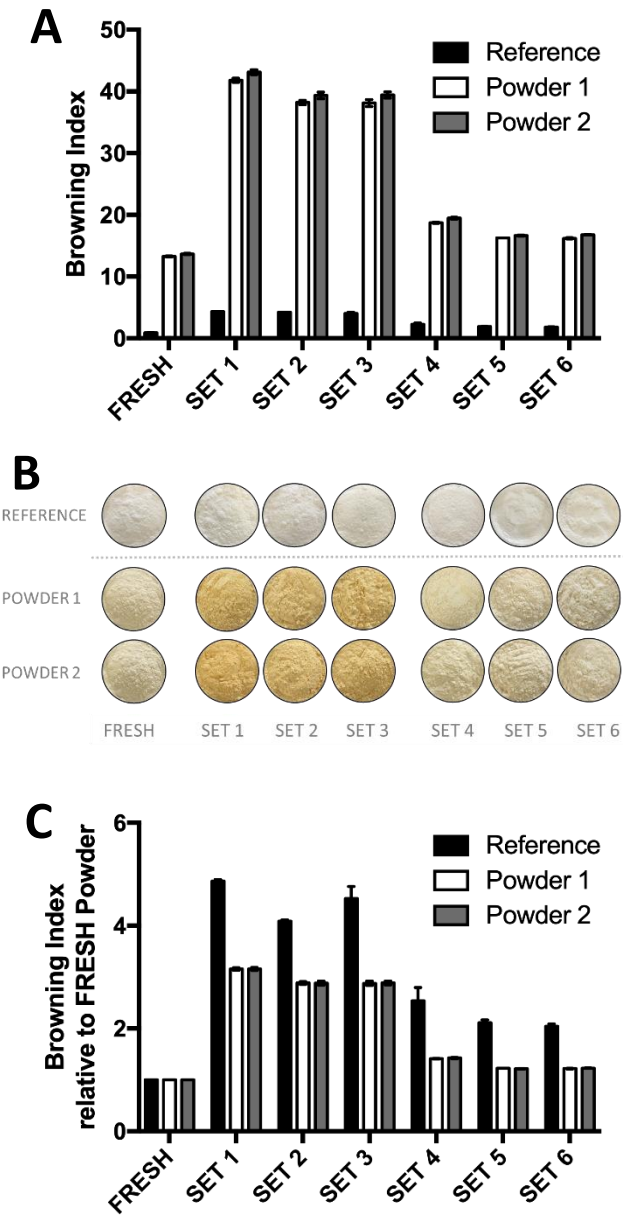


Figure 2: Browning index calculated from $L^* a^* b^*$ for each set. **(A)** Browning index. Mean value obtained for $n = 3$ for each powder. **(B)** Powder appearance during storage. **(C)** Browning index in SET 1 to 6 relative to FRESH powders.

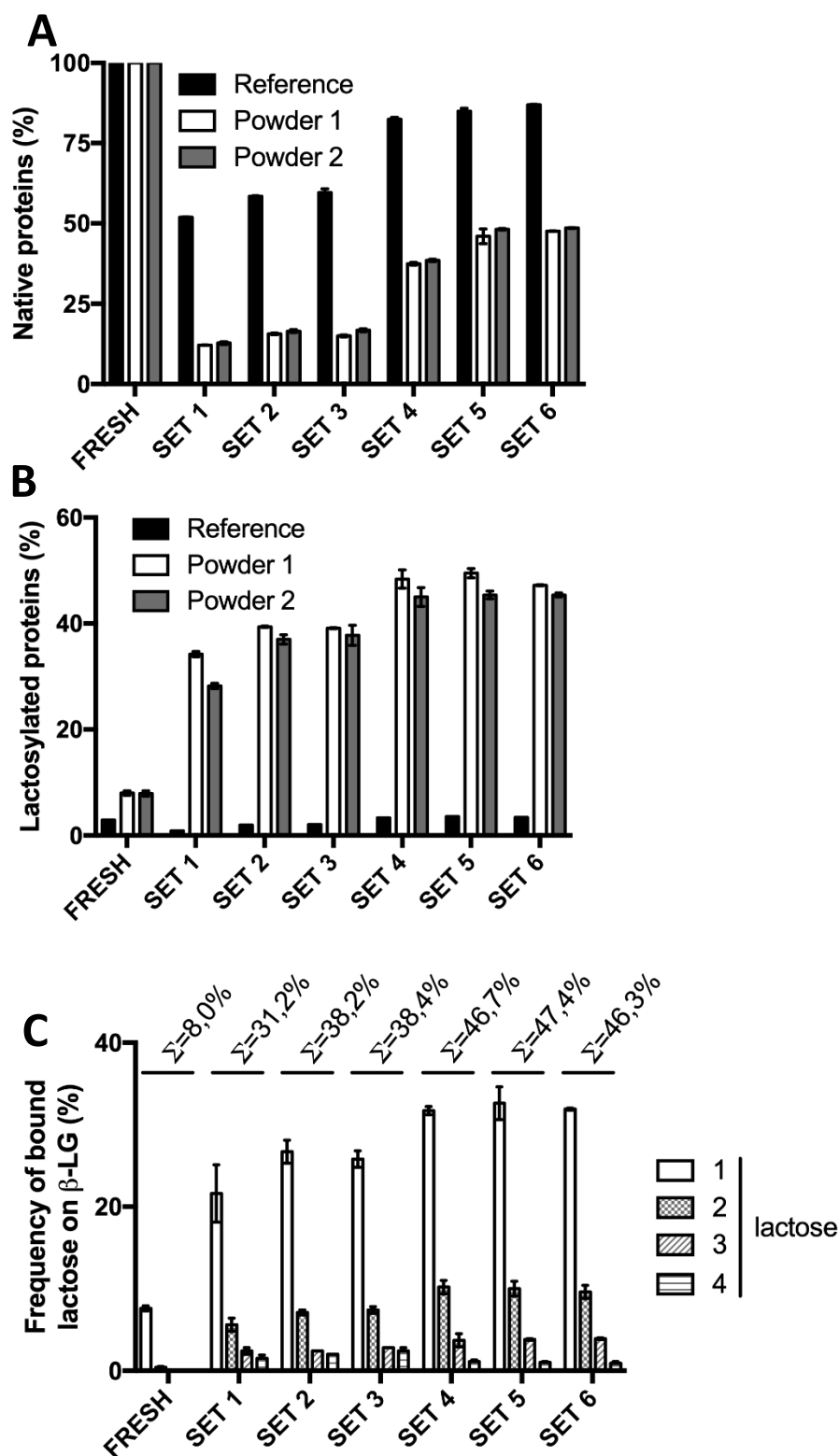


Figure 3: Proportion of native proteins for each storage path determined by RP-HPLC, values for FRESH powders at 100 % are for comparison purpose (A); Protein lactosylation during powder storage (B); Number of lactose associated to lactosylated proteins (mean of powder 1 & 2). Sums of the different lactosylated forms are indicated for each condition (C). Mean value obtained for n = 3 for each powder.

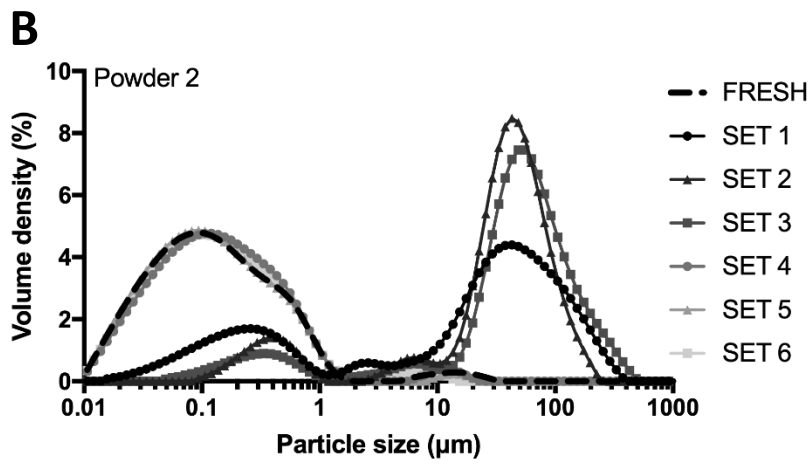
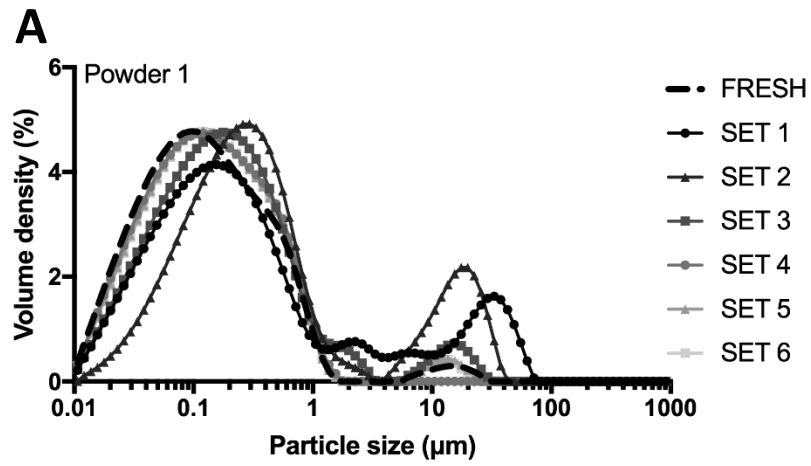


Figure 4: Particle size distribution after powder reconstitution. (A) Powder 1. (B) Powder 2.

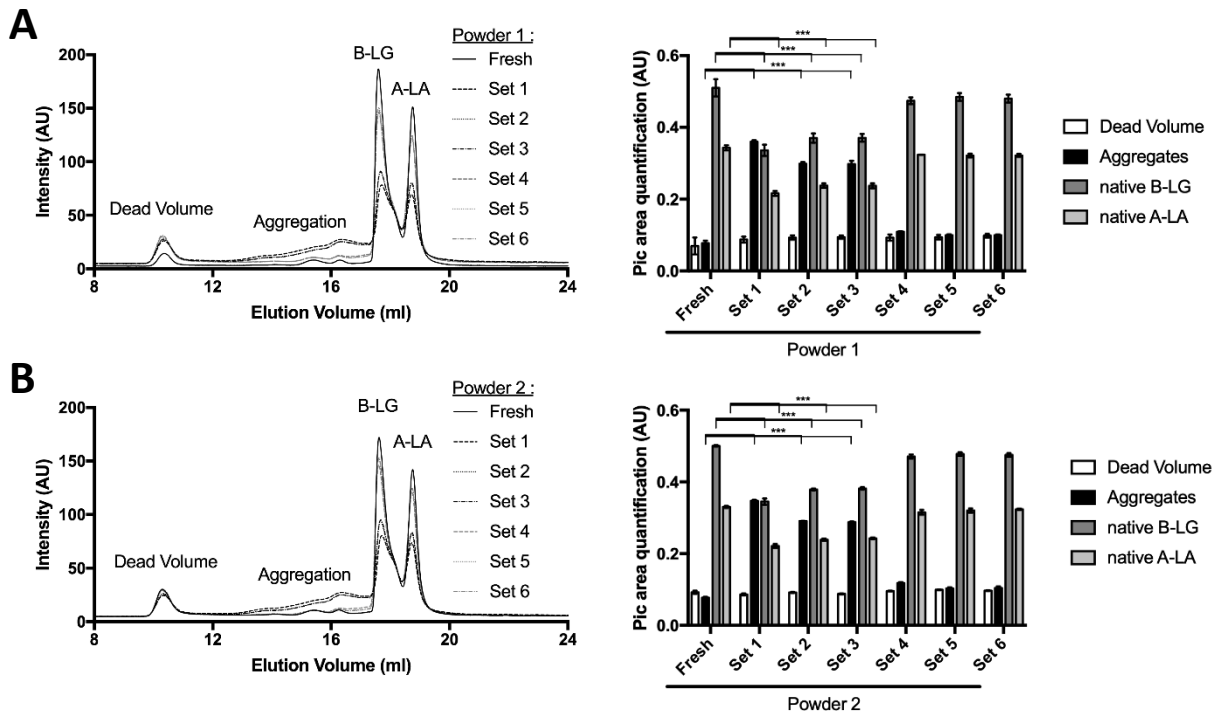


Figure 5: Identification of aggregated proteins during storage with SEC-HPLC. **(A)** Powder 1. **(B)** Powder 2. Proteins and aggregates are separated by size exclusion chromatography (depending on their hydrodynamic radius; e.g. molecular weight for globular proteins; left). Pic integration from chromatograms are represented (right).

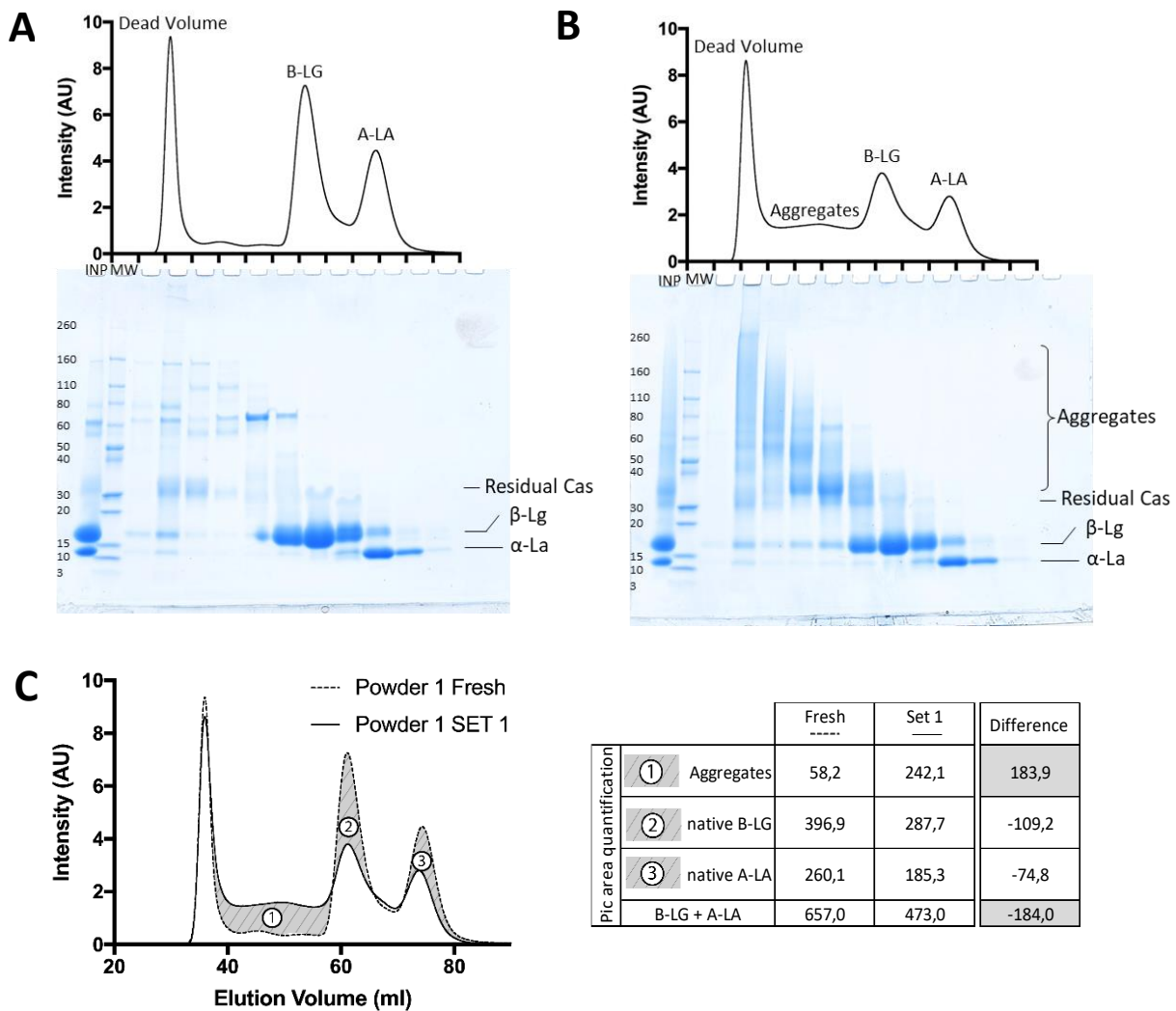


Figure 6: β -lactoglobulin and α -lactalbumin are present in aggregates. Reconstituted and filtered (0,2 μ m) FRESH powder 1 **(A)** and SET 1 Powder 1 **(B)** are separated with size exclusion chromatography and proteins are load on an SDS-PAGE. INP lane represents total milk sample before separation. **(C)** Superposition of FRESH and SET 1 Powder 1 chromatograms (left) and quantification of aggregated and native proteins (right).