



**HAL**  
open science

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

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

### ► To cite this version:

A. Paul, C. Gaiani, L. Cvetkovska, C. Paris, M. Alexander, et al.. Deciphering the impact of whey protein powder storage on protein state and powder stability. *Journal of Food Engineering*, 2022, 326, pp.111050. 10.1016/j.jfoodeng.2022.111050 . hal-03664712

**HAL Id: hal-03664712**

**<https://hal.univ-lorraine.fr/hal-03664712v1>**

Submitted on 4 Oct 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

2

3 A. PAUL<sup>1</sup>, C. GAIANI<sup>1,2</sup>, L. CVETKOVSKA<sup>1</sup>, C. PARIS<sup>1</sup>, M. ALEXANDER<sup>3</sup>, C. RAY<sup>3</sup>, G. FRANCIUS<sup>4</sup>, S.

4 EL-KIRAT-CHATEL<sup>4</sup> & J. BURGAIN<sup>1\*</sup>

5

6 <sup>1</sup> Université de Lorraine, LIBio, F-54000, Nancy, France

7 <sup>2</sup> Institut Universitaire de France (IUF)

8 <sup>3</sup> Arla Foods Ingredients, Sønderupvej 26, 6920 Videbæk, Denmark

9 <sup>4</sup> Université de Lorraine, CNRS, LCPME, F-54000 Nancy, France

10

11 \*Corresponding author: [jennifer.burgain@univ-lorraine.fr](mailto: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

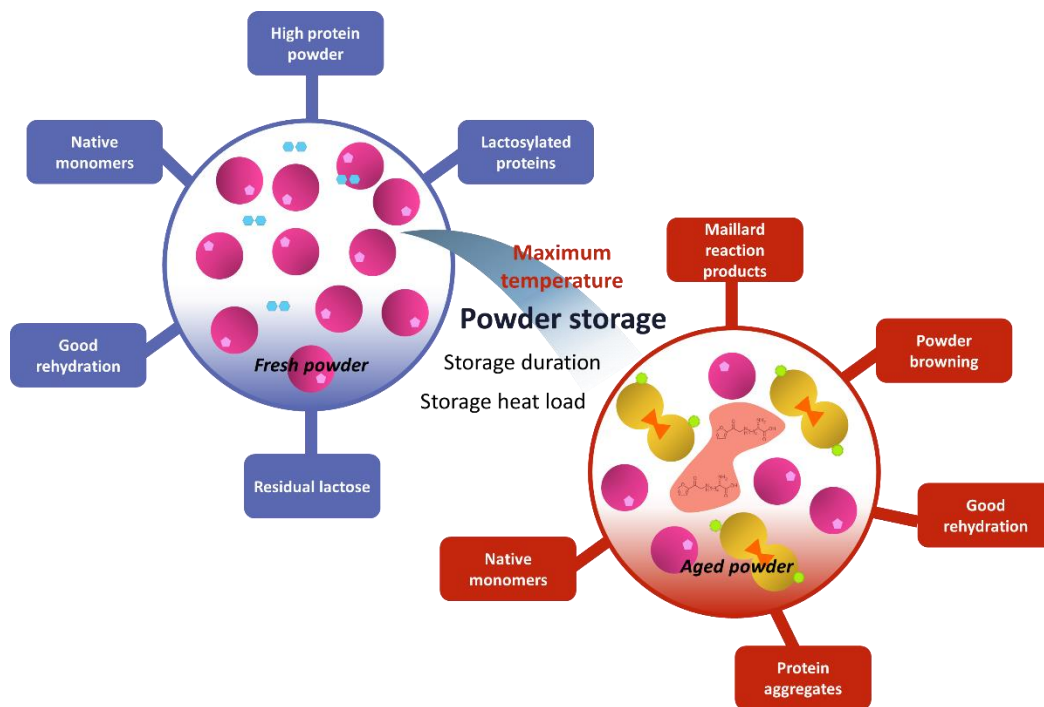
25 browning of the powder, the additional browning that occurs during storage is mostly  
26 independent of lactose content. In addition, during storage, detected lactosylated proteins  
27 were found to be inversely proportional to powder browning indicating the occurrence of  
28 advanced Maillard reaction steps (e.g. dicarbonyl and AGEs formation and production of  
29 melanoidins). Protein aggregates contained both  $\beta$ -lactoglobulin and  $\alpha$ -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,  $\beta$ -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,  $\alpha$ -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 ( $\geq 40$  °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  $\beta$ -Lactoglobulin ( $\beta$ -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  $\mu\text{m}$  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<sup>-1</sup> and the temperature was set at 25 °C. The  
177 pressure was 114 bars and 10  $\mu\text{L}$  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  $\beta$ -LG (A and B variant),  $\alpha$ -lactalbumin ( $\alpha$ -LA), individual caseins ( $\beta$ -casein,  $\alpha$ -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  $\mu$ 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  $\mu$ 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<sup>XL</sup> mass spectrometer equipped with an atmospheric pressure ionization interface  
200 operating in electrospray positive mode (ESI<sup>+</sup>). Ten microliters of sample were separated on a  
201 C18 Alltima reverse phase column (150 x 2.1mm, 5 $\mu$ m – Grace/Alltech, Darmstadt, Germany)  
202 equipped with a C18 Alltima pre-column (7.5 x 2.1mm, 5 $\mu$ m) at 25 °C. The flow rate was set at  
203 0.2 mL.min<sup>-1</sup> 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<sup>-1</sup>) 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<sup>-1</sup> infused in mobile phase (A/B: 50/50) at a flow rate of 5 µL.min<sup>-1</sup>. 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<sup>XL</sup> 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<sup>-1</sup> 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.

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

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  $2.5 \pm 0.1$  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.

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

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.  $\beta$ -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  $\beta$ -LG (maximum detected = 4 lactose /  $\beta$ -LG) is  
374 poor considering that up to eleven lactose could theoretically be bound to  $\beta$ -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**,  $\beta$ -lactoglobulin and  $\alpha$ -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  $\beta$ -lactoglobulin and  $\alpha$ -  
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  $\beta$ -lactoglobulin and  
419  $\alpha$ -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  $\beta$ -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  $\beta$ -  
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  $\beta$ -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  $\beta$ -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,  $\beta$ -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  $\beta$ -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  $\beta$ -lactoglobulin and  $\alpha$ -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  $\beta$ -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

## 560 6. Acknowledgements

561 The authors acknowledge support of the LIBio project of the "Lorraine Université d'Excellence"  
562 (Investissements d'avenir – ANR 15-004). We thank the PASM platform (Structural and  
563 Metabolomic Analysis Platform, SF4242, EFABA, University of Lorraine, Vandoeuvre-lès-Nancy,  
564 France) for the use of the Tribrid Orbitrap ID-X Mass Spectrometer.

565

## 566 7. Conflict of interest

567 MA and CR work for ARLA Foods Ingredients Group. No potential conflict of interest was  
568 reported by the other authors.

569

## 570 8. References

571 AFNOR. (2004). *Microbiology of food and animal feeding stuffs—Determination of water activity, ISO*  
572 *21807:2004*.

573 Andrewes, P. (2022). Predicting the shelf-life of microbially-stabilised dairy products: What are the  
574 roles of stability studies, storage trials, 'accelerated' trials, and dairy science? *International*  
575 *Dairy Journal*, 125, 105239. <https://doi.org/10.1016/j.idairyj.2021.105239>

576 Anema, S. G., Pinder, D. N., Hunter, R. J., & Hemar, Y. (2006). Effects of storage temperature on the  
577 solubility of milk protein concentrate (MPC85). *Food Hydrocolloids*, 20(2), 386–393.  
578 <https://doi.org/10.1016/j.foodhyd.2005.03.015>

579 Baisier, W. M., & Labuza, T. P. (1992). Maillard browning kinetics in a liquid model system. *Journal of*  
580 *Agricultural and Food Chemistry*, 40(5), 707–713. <https://doi.org/10.1021/jf00017a001>

581 Barroso da Silva, F. L., Carloni, P., Cheung, D., Cottone, G., Donnini, S., Foegeding, E. A., Gulzar, M.,  
582 Jacquier, J. C., Lobaskin, V., MacKernan, D., Mohammad Hosseini Naveh, Z., Radhakrishnan,  
583 R., & Santiso, E. E. (2020). Understanding and controlling food protein structure and function  
584 in foods: Perspectives from experiments and computer simulations. *Annual Review of Food  
585 Science and Technology*, 11(1), 365–387. [https://doi.org/10.1146/annurev-food-032519-  
586 051640](https://doi.org/10.1146/annurev-food-032519-051640)

587 Boekel, M. A. J. S. van. (2001). Kinetic aspects of the Maillard reaction: A critical review. *Food /  
588 Nahrung*, 45(3), 150–159. [https://doi.org/10.1002/1521-3803\(20010601\)45:3<150::AID-  
589 FOOD150>3.0.CO;2-9](https://doi.org/10.1002/1521-3803(20010601)45:3<150::AID-FOOD150>3.0.CO;2-9)

590 Capuano, E., Oliviero, T., & van Boekel, M. A. J. S. (2018). Modeling food matrix effects on chemical  
591 reactivity: Challenges and perspectives. *Critical Reviews in Food Science and Nutrition*,  
592 58(16), 2814–2828. <https://doi.org/10.1080/10408398.2017.1342595>

593 Chen, Z., Leinisch, F., Greco, I., Zhang, W., Shu, N., Chuang, C. Y., Lund, M. N., & Davies, M. J. (2019).  
594 Characterisation and quantification of protein oxidative modifications and amino acid  
595 racemisation in powdered infant milk formula. *Free Radical Research*, 53(1), 68–81.  
596 <https://doi.org/10.1080/10715762.2018.1554250>

597 Crowley, S. V., Kelly, A. L., Schuck, P., Jeantet, R., & O'Mahony, J. A. (2016). Rehydration and Solubility  
598 Characteristics of High-Protein Dairy Powders. In P. L. H. McSweeney & J. A. O'Mahony (Eds.),  
599 *Advanced Dairy Chemistry: Volume 1B: Proteins: Applied Aspects* (pp. 99–131). Springer.  
600 [https://doi.org/10.1007/978-1-4939-2800-2\\_4](https://doi.org/10.1007/978-1-4939-2800-2_4)

601 Fan, F., & Roos, Y. H. (2015). X-ray diffraction analysis of lactose crystallization in freeze-dried  
602 lactose–whey protein systems. *Food Research International*, 67, 1–11.  
603 <https://doi.org/10.1016/j.foodres.2014.10.023>

604 Ferrer, E., Alegría, A., Farré, R., Abellán, P., Romero, F., & Clemente, G. (2003). Evolution of available  
605 lysine and furosine contents in milk-based infant formulas throughout the shelf-life storage

606 period. *Journal of the Science of Food and Agriculture*, 83(5), 465–472.  
607 <https://doi.org/10.1002/jsfa.1402>

608 Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification  
609 of total lipides from animal tissues. *The Journal of Biological Chemistry*, 226(1), 497–509.

610 Forny, L., Marabi, A., & Palzer, S. (2011). Wetting, disintegration and dissolution of agglomerated  
611 water soluble powders. *Powder Technology*, 206(1), 72–78.  
612 <https://doi.org/10.1016/j.powtec.2010.07.022>

613 Gaiani, C., Scher, J., Schuck, P., Desobry, S., & Banon, S. (2009). Use of a turbidity sensor to determine  
614 dairy powder rehydration properties. *Powder Technology*, 190(1), 2–5.  
615 <https://doi.org/10.1016/j.powtec.2008.04.042>

616 Gasparini, A., Van Gool, M. P., Bultsma, M., Cutroneo, S., Sforza, S., & Tedeschi, T. (2020).  
617 Modifications induced by controlled storage conditions on whey protein concentrates:  
618 Effects on whey protein lactosylation and solubility. *International Dairy Journal*, 109, 104765.

619 Gazi, I., & Huppertz, T. (2015). Influence of protein content and storage conditions on the solubility of  
620 caseins and whey proteins in milk protein concentrates. *International Dairy Journal*, 46, 22–  
621 30. <https://doi.org/10.1016/j.idairyj.2014.09.009>

622 Gulzar, M., Bouhallab, S., Jeantet, R., Schuck, P., & Croguennec, T. (2011). Influence of pH on the dry  
623 heat-induced denaturation/aggregation of whey proteins. *Food Chemistry*, 129(1), 110–116.  
624 <https://doi.org/10.1016/j.foodchem.2011.04.037>

625 Guyomarc’h, F., Famelart, M.-H., Henry, G., Gulzar, M., Leonil, J., Hamon, P., Bouhallab, S., &  
626 Croguennec, T. (2015). Current ways to modify the structure of whey proteins for specific  
627 functionalities—A review. *Dairy Science & Technology*, 95(6), 795–814.  
628 <https://doi.org/10.1007/s13594-014-0190-5>

629 Guyomarc’h, F., Warin, F., Donald Muir, D., & Leaver, J. (2000). Lactosylation of milk proteins during  
630 the manufacture and storage of skim milk powders. *International Dairy Journal*, 10(12), 863–  
631 872. [https://doi.org/10.1016/S0958-6946\(01\)00020-6](https://doi.org/10.1016/S0958-6946(01)00020-6)

632 Havea, P. (2006). Protein interactions in milk protein concentrate powders. *International Dairy*  
633 *Journal*, 16(5), 415–422.

634 Hiller, B., & Lorenzen, P. C. (2010). Functional properties of milk proteins as affected by Maillard  
635 reaction induced oligomerisation. *Food Research International*, 43(4), 1155–1166.  
636 <https://doi.org/10.1016/j.foodres.2010.02.006>

637 Hodge, J. E. (1953). Dehydrated foods, chemistry of browning reactions in model systems. *Journal of*  
638 *Agricultural and Food Chemistry*, 1(15), 928–943. <https://doi.org/10.1021/jf60015a004>

639 IDF. (1979). Determination of the dispersibility and wettability of instant dried milk. *International*  
640 *Dairy Federation, Brussels, Belgium*.

641 IDF. (2005). *Dried milk and dried milk products—Determination of insolubility index*.

642 Ji, J., Fitzpatrick, J., Cronin, K., Crean, A., & Miao, S. (2016). Assessment of measurement  
643 characteristics for rehydration of milk protein based powders. *Food Hydrocolloids*, 54, 151–  
644 161. <https://doi.org/10.1016/j.foodhyd.2015.09.027>

645 Kelly, P. (2019). Chapter 3 - Manufacture of whey protein products: Concentrates, isolate, whey  
646 protein fractions and microparticulated. In H. C. Deeth & N. Bansal (Eds.), *Whey Proteins* (pp.  
647 97–122). Academic Press. <https://doi.org/10.1016/B978-0-12-812124-5.00003-5>

648 Kern, L., Fabre, O., Scher, J., & Petit, J. (2020). Chemical fractionation of caseins by differential  
649 precipitation: Influence of pH, calcium addition, protein concentration and temperature on  
650 the depletion in  $\alpha$ - and  $\beta$ -caseins. *International Journal of Food Science & Technology*, 55(2),  
651 542–552. <https://doi.org/10.1111/ijfs.14283>

652 Le, T. T., Bhandari, B., Holland, J. W., & Deeth, H. C. (2011). Maillard reaction and protein cross-  
653 linking in relation to the solubility of milk powders. *Journal of Agricultural and Food*  
654 *Chemistry*, 59(23), 12473–12479. <https://doi.org/10.1021/jf203460z>

655 Le, T. T., Holland, J. W., Bhandari, B., Alewood, P. F., & Deeth, H. C. (2013). Direct evidence for the  
656 role of Maillard reaction products in protein cross-linking in milk powder during storage.  
657 *International Dairy Journal*, 31(2), 83–91. <https://doi.org/10.1016/j.idairyj.2013.02.013>

658 Leclère, J., & Birlouez-Aragon, I. (2001). The fluorescence of advanced Maillard products is a good  
659 indicator of lysine damage during the Maillard reaction. *Journal of Agricultural and Food*  
660 *Chemistry*, 49(10), 4682–4687. <https://doi.org/10.1021/jf001433o>

661 Leiva, G. E., Naranjo, G. B., & Malec, L. S. (2017). A study of different indicators of Maillard reaction  
662 with whey proteins and different carbohydrates under adverse storage conditions. *Food*  
663 *Chemistry*, 215, 410–416. <https://doi.org/10.1016/j.foodchem.2016.08.003>

664 Lin, R., Wang, Y., & Selomulya, C. (2022). Physical Properties of Dairy Powders. In P. L. H. McSweeney  
665 & J. P. McNamara (Eds.), *Encyclopedia of Dairy Sciences (Third Edition)* (pp. 504–520).  
666 Academic Press. <https://doi.org/10.1016/B978-0-12-818766-1.00028-3>

667 Linetsky, M., Hill, J. M. W., LeGrand, R. D., & Hu, F. (2004). Dehydroalanine crosslinks in human lens.  
668 *Experimental Eye Research*, 79(4), 499–512. <https://doi.org/10.1016/j.exer.2004.06.026>

669 Liu, G., & Zhong, Q. (2013). Thermal aggregation properties of whey protein glycated with various  
670 saccharides. *Food Hydrocolloids*, 32(1), 87–96.  
671 <https://doi.org/10.1016/j.foodhyd.2012.12.008>

672 Marvin, L. F., Parisod, V., Fay, L. B., & Guy, P. A. (2002). Characterization of lactosylated proteins of  
673 infant formula powders using two-dimensional gel electrophoresis and nanoelectrospray  
674 mass spectrometry. *Electrophoresis*, 23(15), 2505–2512.

675 Maubois, J.-L., & Lorient, D. (2016). Dairy proteins and soy proteins in infant foods nitrogen-to-  
676 protein conversion factors. *Dairy Science & Technology*, 96(1), 15–25.  
677 <https://doi.org/10.1007/s13594-015-0271-0>

678 Mehta, B. M., & Deeth, H. C. (2016). Blocked lysine in dairy products: Formation, occurrence,  
679 analysis, and nutritional implications. *Comprehensive Reviews in Food Science and Food*  
680 *Safety*, 15(1), 206–218. <https://doi.org/10.1111/1541-4337.12178>

681 Mimouni, A., Deeth, H. C., Whittaker, A. K., Gidley, M. J., & Bhandari, B. R. (2010). Investigation of the  
682 microstructure of milk protein concentrate powders during rehydration: Alterations during  
683 storage. *Journal of Dairy Science*, 93(2), 463–472. <https://doi.org/10.3168/jds.2009-2369>

684 Morales, F. J., & van Boekel, M. A. J. S. (1998). A study on advanced Maillard reaction in heated  
685 casein/sugar solutions: Colour formation. *International Dairy Journal*, 8(10), 907–915.  
686 [https://doi.org/10.1016/S0958-6946\(99\)00014-X](https://doi.org/10.1016/S0958-6946(99)00014-X)

687 Morgan, F., Bouhallab, S., Mollé, D., Henry, G., Maubois, J.-L., & Léonil, J. (1998). Lactolation of  $\beta$ -  
688 Lactoglobulin monitored by electrospray ionisation mass spectrometry. *International Dairy*  
689 *Journal*, 8(2), 95–98. [https://doi.org/10.1016/S0958-6946\(98\)00025-9](https://doi.org/10.1016/S0958-6946(98)00025-9)

690 Morgan, F., Henry, G., Le Graët, Y., Mollé, D., Léonil, J., & Bouhallab, S. (1999). Resistance of  $\beta$ -  
691 lactoglobulin-bound lactose to the hydrolysis by  $\beta$ -galactosidase. *International Dairy Journal*,  
692 9(11), 813–816.

693 Mortensen, A. B., & Wallin, H. (1989). Gravimetric Determination of Ash in Foods: NMKL  
694 Collaborative Study. *Journal of Association of Official Analytical Chemists*, 72(3), 481–483.  
695 <https://doi.org/10.1093/jaoac/72.3.481>

696 Nasser, S., Moreau, A., Jeantet, R., Hédoux, A., & Delaplace, G. (2017). Influence of storage  
697 conditions on the functional properties of micellar casein powder. *Food and Bioproducts*  
698 *Processing*, 106, 181–192. <https://doi.org/10.1016/j.fbp.2017.09.004>

699 *NF EN ISO 8968-1*. (2014). Afnor EDITIONS. [https://www.boutique.afnor.org/fr-fr/norme/nf-en-iso-](https://www.boutique.afnor.org/fr-fr/norme/nf-en-iso-89681/lait-et-produits-laitiers-determination-de-la-teneur-en-azote-partie-1-meth/fa129096/43035)  
700 [89681/lait-et-produits-laitiers-determination-de-la-teneur-en-azote-partie-1-](https://www.boutique.afnor.org/fr-fr/norme/nf-en-iso-89681/lait-et-produits-laitiers-determination-de-la-teneur-en-azote-partie-1-meth/fa129096/43035)  
701 [meth/fa129096/43035](https://www.boutique.afnor.org/fr-fr/norme/nf-en-iso-89681/lait-et-produits-laitiers-determination-de-la-teneur-en-azote-partie-1-meth/fa129096/43035)

702 Nicolai, T., Britten, M., & Schmitt, C. (2011).  $\beta$ -Lactoglobulin and WPI aggregates: Formation,  
703 structure and applications. *Food Hydrocolloids*, 25(8), 1945–1962.  
704 <https://doi.org/10.1016/j.foodhyd.2011.02.006>

705 Norwood, E.-A., Chevallier, M., Le Floch-Fouéré, C., Schuck, P., Jeantet, R., & Croguennec, T. (2016).  
706 Heat-induced aggregation properties of whey proteins as affected by storage conditions of  
707 whey protein isolate powders. *Food and Bioprocess Technology*, 9(6), 993–1001.  
708 <https://doi.org/10.1007/s11947-016-1686-1>

709 Norwood, E.-A., Pezennec, S., Burgain, J., Briard-Bion, V., Schuck, P., Croguennec, T., Jeantet, R., & Le  
710 Floch-Fouéré, C. (2017). Crucial role of remaining lactose in whey protein isolate powders  
711 during storage. *Journal of Food Engineering*, *195*, 206–216.  
712 <https://doi.org/10.1016/j.jfoodeng.2016.10.010>

713 Price, J. (2019). Chapter 2—History of the development and application of whey protein products. In  
714 H. C. Deeth & N. Bansal (Eds.), *Whey Proteins* (pp. 51–95). Academic Press.  
715 <https://doi.org/10.1016/B978-0-12-812124-5.00002-3>

716 Schuck, P., Dolivet, A., & Jeantet, R. (2012). *Analytical methods for food and dairy powders*. John  
717 Wiley and Sons. <https://hal.archives-ouvertes.fr/hal-01209392>

718 Thomas, M. E. C., Scher, J., & Desobry, S. (2005). Study of lactose /  $\beta$ -lactoglobulin interactions during  
719 storage. *Lait*, *85*(4–5), 325–333. <https://doi.org/10.1051/lait:2005013>

720 Van Boekel, M. (1998). Effect of heating on Maillard reactions in milk. *Food Chemistry*, *62*(4), 403–  
721 414.

722 Van Boekel, M. A. J. S. (2008). Kinetic Modeling of Food Quality: A Critical Review. *Comprehensive*  
723 *Reviews in Food Science and Food Safety*, *7*(1), 144–158. [https://doi.org/10.1111/j.1541-](https://doi.org/10.1111/j.1541-4337.2007.00036.x)  
724 [4337.2007.00036.x](https://doi.org/10.1111/j.1541-4337.2007.00036.x)

725 van Boekel, M. A. J. S. (2020). On the pros and cons of Bayesian kinetic modeling in food science.  
726 *Trends in Food Science & Technology*, *99*, 181–193.  
727 <https://doi.org/10.1016/j.tifs.2020.02.027>

728 Wooster, T. J., & Augustin, M. A. (2007). Rheology of whey protein–dextran conjugate films at the  
729 air/water interface. *Food Hydrocolloids*, *21*(7), 1072–1080.  
730 <https://doi.org/10.1016/j.foodhyd.2006.07.015>

731 Zhou, P., & Labuza, T. P. (2007). Effect of water content on glass transition and protein aggregation of  
732 whey protein powders during short-term storage. *Food Biophysics*, *2*(2), 108–116.  
733 <https://doi.org/10.1007/s11483-007-9037-4>

734



735

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

736

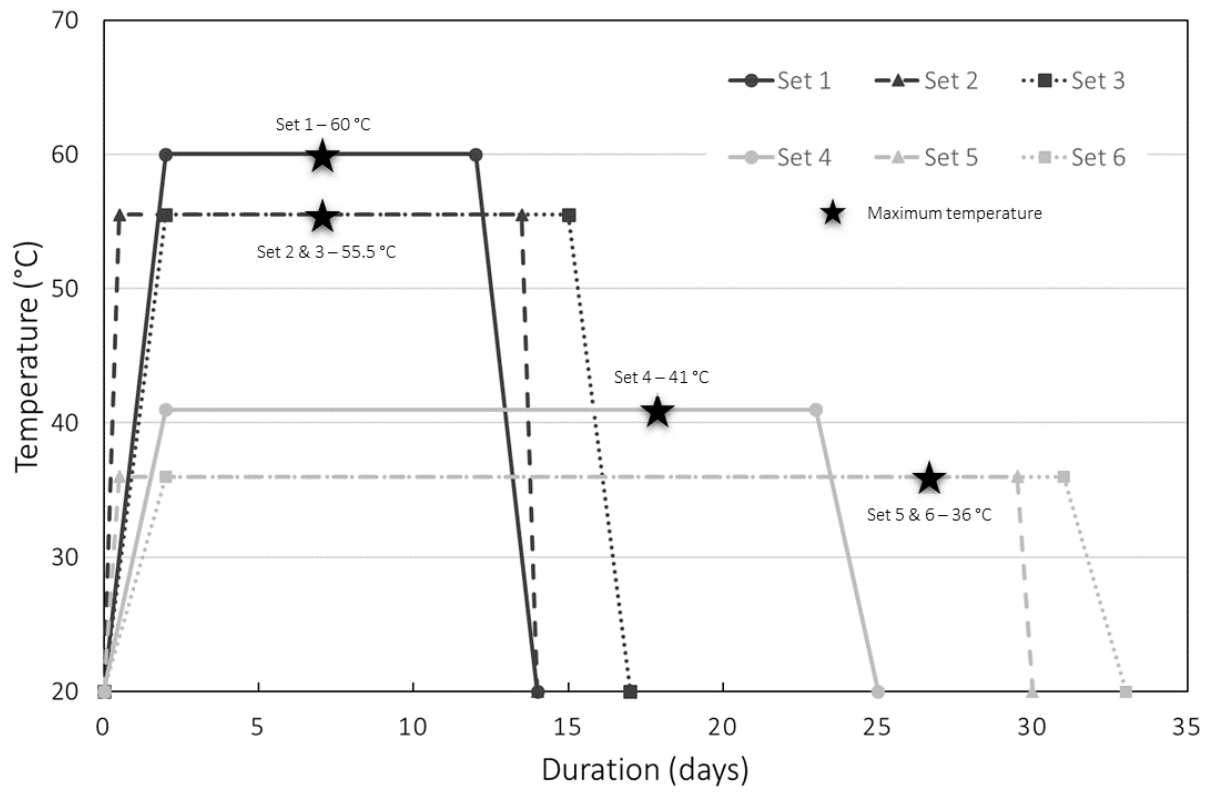
	Powder 1	Powder 2	Reference
Lipids (g.100g <sup>-1</sup> )	6.4 ± 0.4	4.8 ± 0.0	-
Lactose* (g.100g <sup>-1</sup> )	1.5	1.5	-
Ashes (g.100g <sup>-1</sup> )	2.8 ± 0.0	2.7 ± 0.1	2.3 ± 0.1
Water (g.100g <sup>-1</sup> )	5.0 ± 0.0	4.3 ± 0.1	4.6 ± 0.1
<b>Total proteins (g.100g<sup>-1</sup>)</b>	<b>79.6 ± 0.1</b>	<b>84.1 ± 1.1</b>	<b>83.9 ± 3.9</b>
<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	-

737

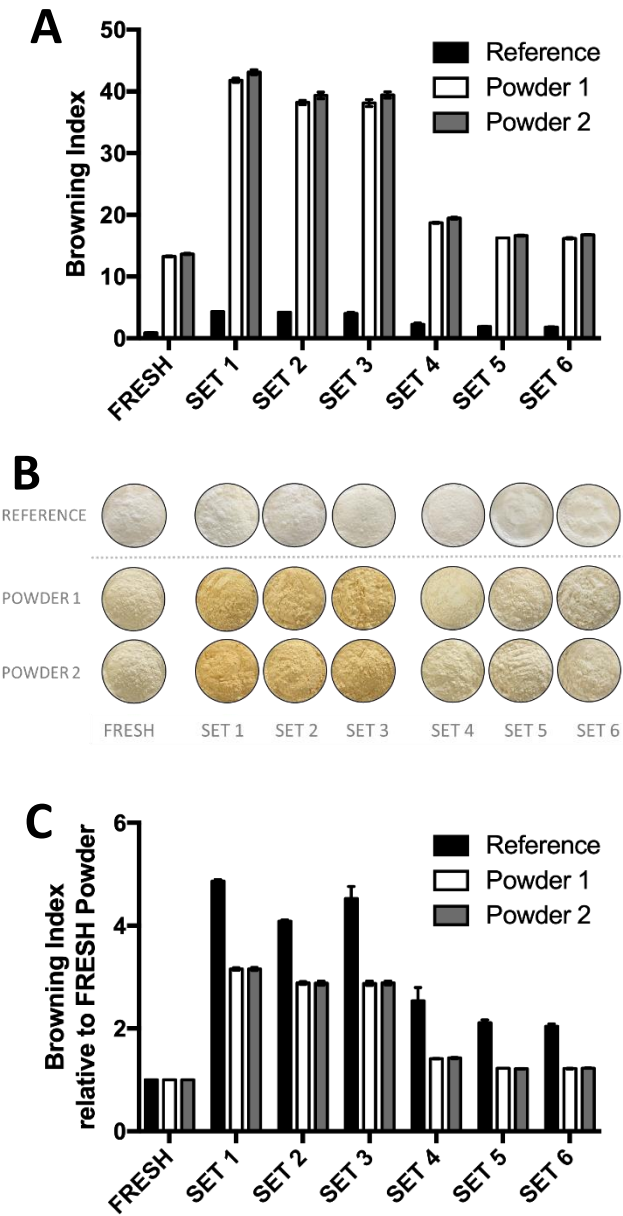
\*Data provided by AFI

738

739



**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.

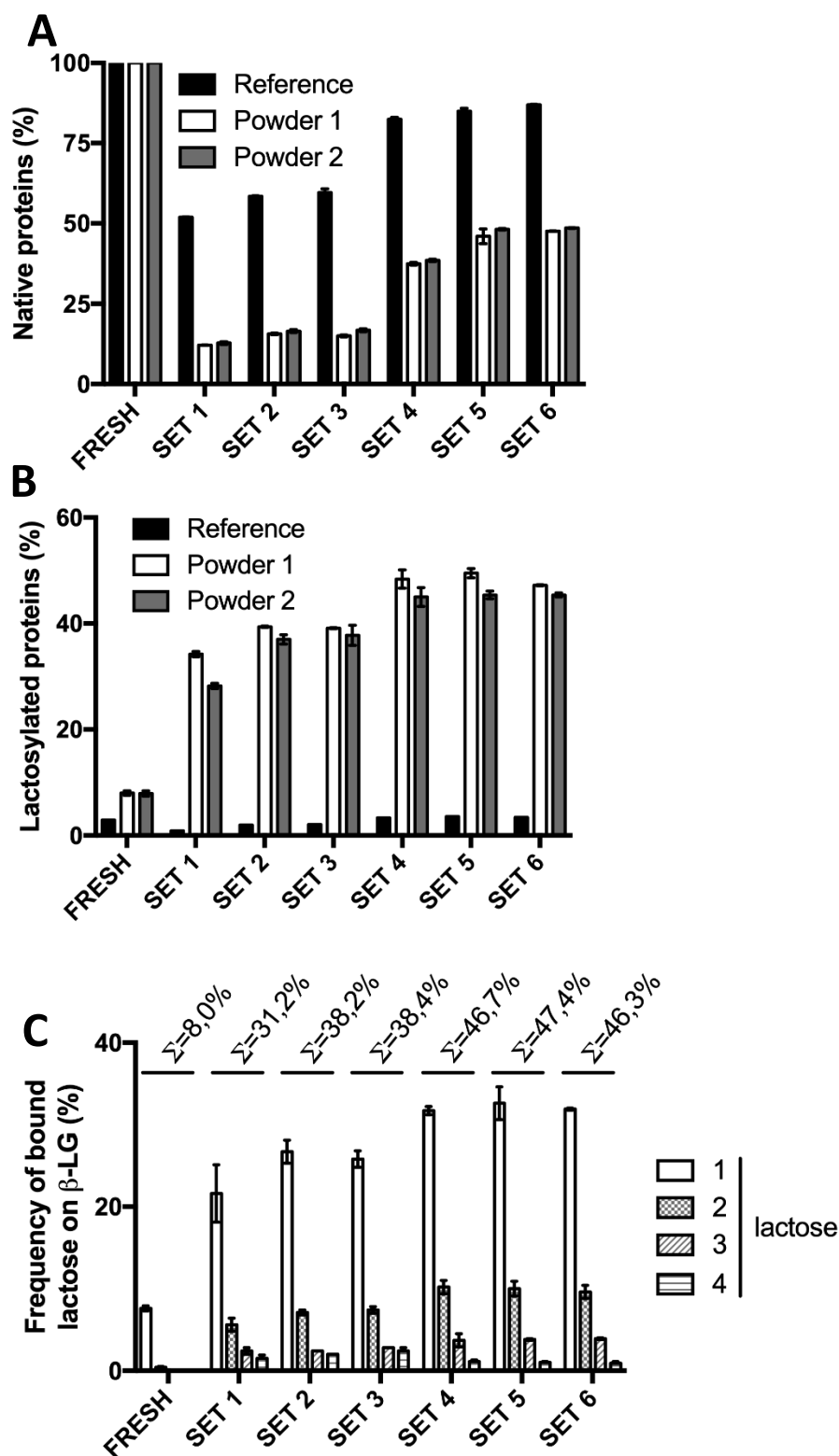


Figure 3: Proportion of native proteins for each storage path determined by RP-HPLC, values for FRESH powders at 100 % are for comparison purpose (A); Protein lactosylation during powder storage (B); Number of lactose associated to lactosylated proteins (mean of powder 1 & 2). Sums of the different lactosylated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

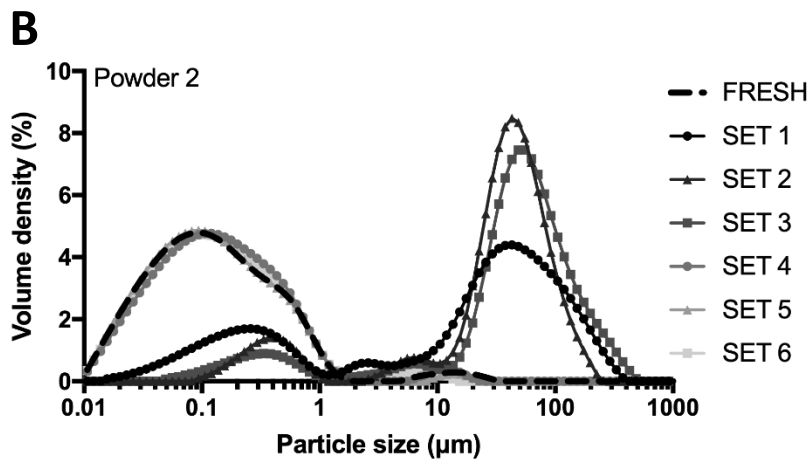
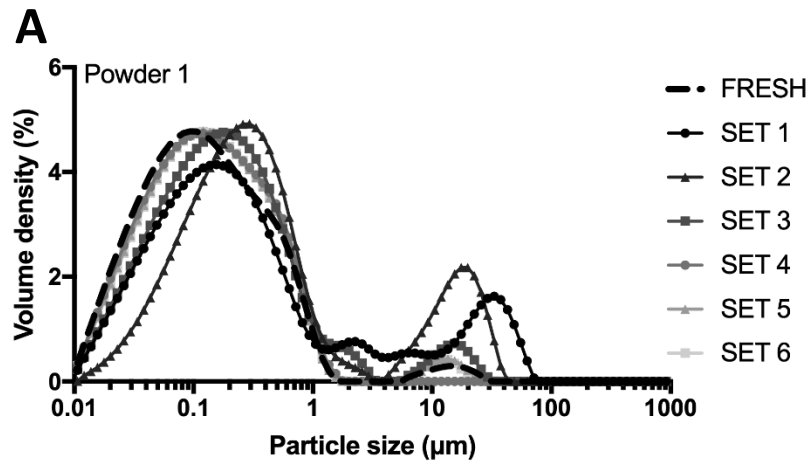
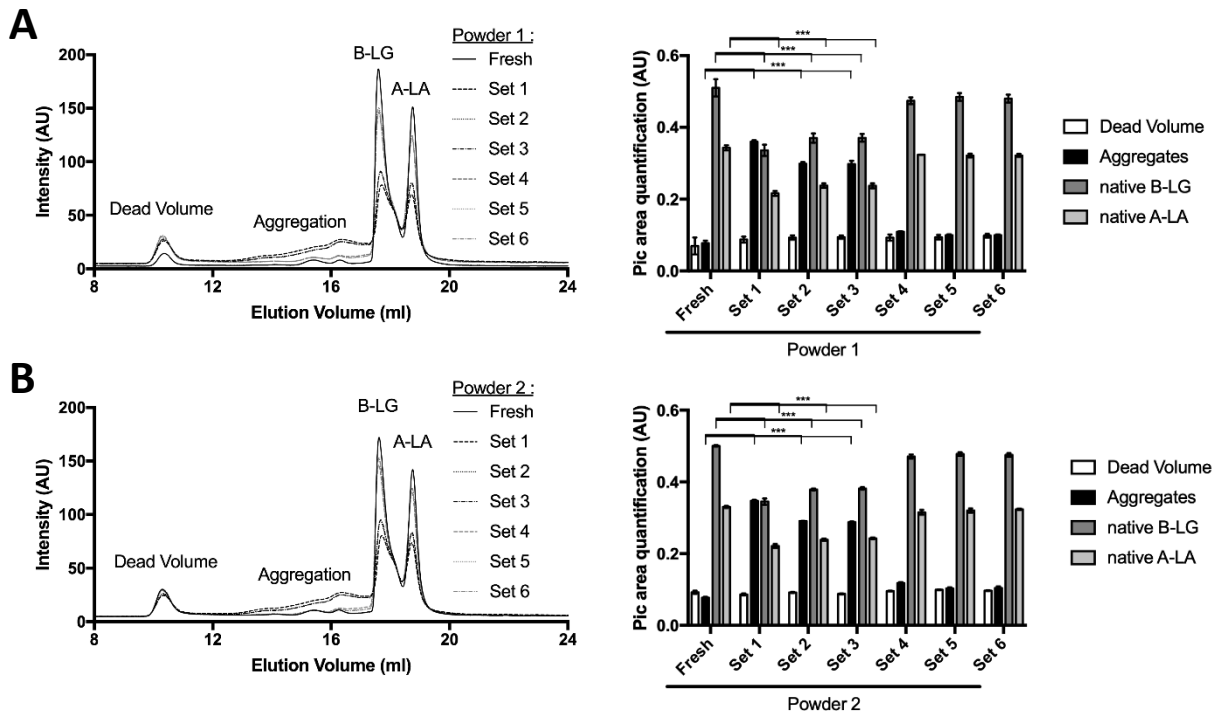
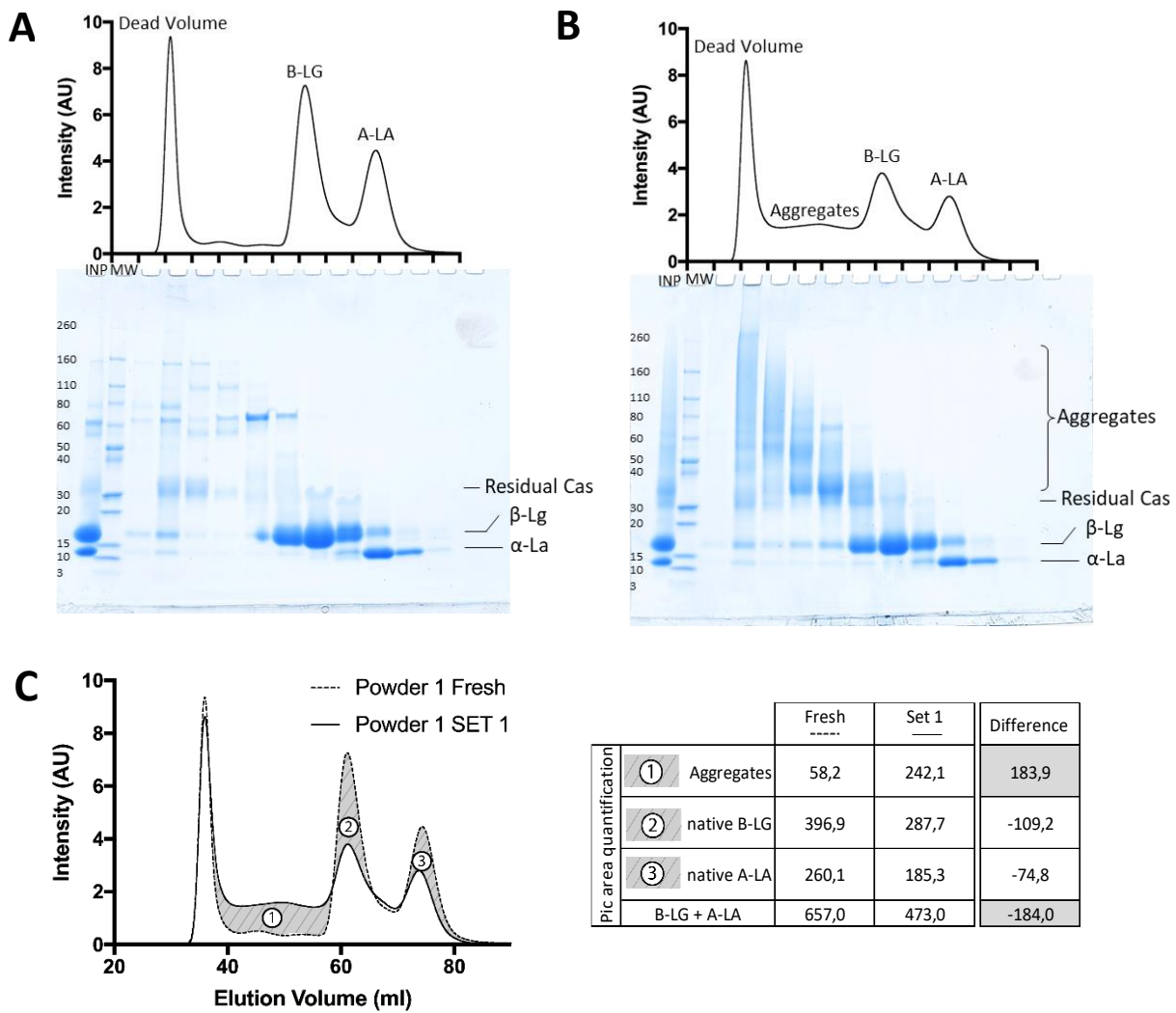


Figure 4: Particle size distribution after powder reconstitution. (A) Powder 1. (B) Powder 2.



**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:**  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin are present in aggregates. Reconstituted and filtered (0,2 $\mu$ 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).