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

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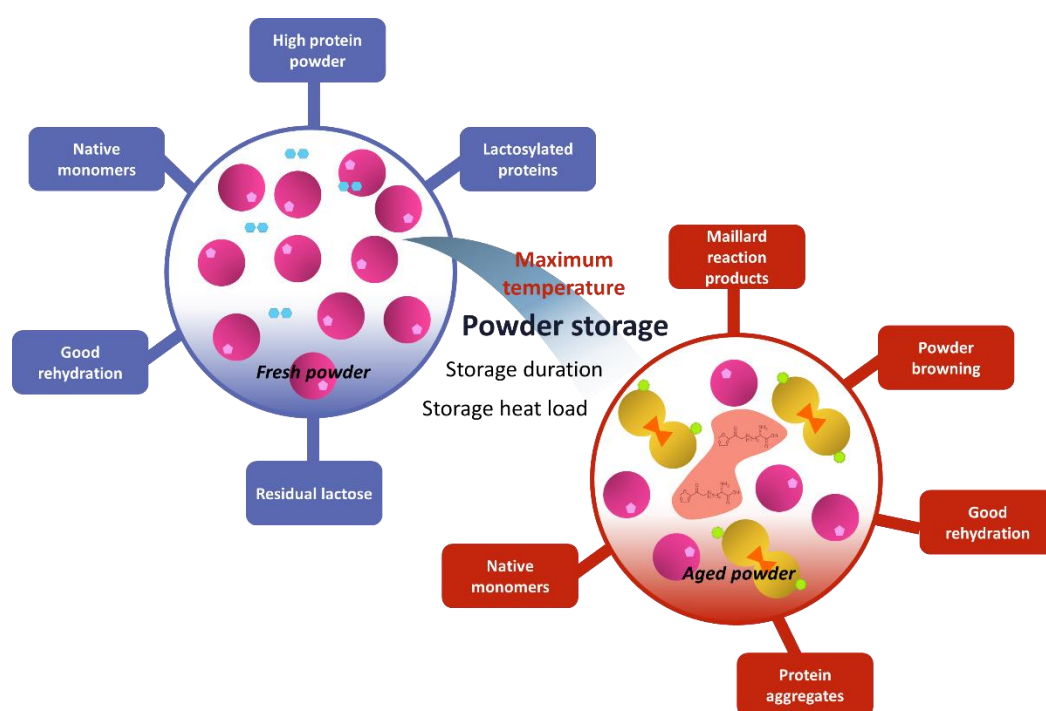
## Abstract

Whey protein concentrate powders can be strongly affected by storage conditions inducing both chemical and structural protein modifications. In the present work, a combination of different storage conditions were evaluated, leading to the evaluation of 6 different batches that were chosen in order to evaluate the impact of the following parameters: storage temperature, storage duration and storage heat load (°C.days). In each set the relative humidity was maintained at 70%. To estimate the impact of the three parameters, whey protein lactosylation, denaturation and aggregation were followed. It was shown that the highest temperatures (60 vs 55.5 °C and 41 vs 36 °C) led to greater protein modification, whereas the duration (17 vs 14 days and 33 vs 30 days) and the total heat load (low vs high C°.days) did not affect protein modification. Lactose content is known to be an important parameter for powder browning. Here, we show that if lactose content is crucial for the level of initial process induced

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

**Key words:** dairy powders, whey proteins,  $\beta$ -Lactoglobulin, lactosylation, aggregation, Maillard reaction, storage

## Graphical abstract



## 1. Introduction

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

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

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

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

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

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

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

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

## **2. Material and methods**

### **2.1. Dairy powders production**

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

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

## 2.2 Powder storage conditions

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

## 2.3 Powder composition

### 2.3.1 Water content

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

### 2.3.2 Proteins

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

### 2.3.3 Lipids

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

### 2.3.4 Minerals

Mineral content was performed by mineralising 2 g of sample, and drying at 550 °C during 12 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  $\mu$ 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  $\mu$ 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

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

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

#### 2.4.2 Protein lactosylation (using UHPLC-MS)

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

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

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

Ion optics parameters were optimized by automatic tuning using a standard solution of BSA at 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 screening of total soluble proteins was realized using UV230 absorbance. Structural elucidation of the different lactosylated forms of protein was realized thanks to full scan MS spectra performed on LTQ<sup>XL</sup> analyzer from 1500 to 4000 m/z, and subsequent assignment of charge states of proteins.

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

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

The samples were rehydrated a day before at 1% (m/v) at 4 °C in ultra-pure water. A centrifugation 2 min at 14500 rpm was followed by 0,2 µm filtration. For HPLC-SEC analyses (Shimadzu Corporation, Kyoto, Japan, see section 2.4.1 for more details), 80 µg of protein are injected in a series of Agilent Bio SEC-5, 150 Å, 7.8 x 300 mm, 5 µm column, followed by Agilent Bio SEC-5, 400 Å, 7.8 x 300 mm, 5 µm preequilibrated in PBS 1X pH 6.8. Each run is performed at 25°C at 1mL/min during 30 minutes and all samples are performed in triplicates. Data and pics integration are obtained at 280 nm with the Shimadzu LC solution software. For low 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  $103\text{ }^{\circ}\text{C}$

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

#### 2.5.4 Size of aggregates

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

#### 2.5.5 Color measurements

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

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

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

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

### 2.7. Statistical analysis

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

### 3. Results

#### 3.1. Evolution of powder functional properties during storage

##### 3.1.1. Powder composition

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

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

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

### ***3.1.2. Powder color***

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

### ***3.1.3. Powder rehydration***



It is commonly believed that the rehydration process consists of three sequential stages, which are wetting, dispersion and solubilisation (Crowley et al., 2016). Wetting is the first step as the particles come in contact with the liquid, while dispersion and solubilisation are the critical phases, when primary particles start to release material from the particle surface into the liquid (Forny et al., 2011; Ji et al., 2016). The evaluation of powder functional properties to describe rehydration ability was done with IDF standards. The results for solubility and dispersibility are presented in **Table S2**. All the powders in the present study were considered non-wettable (time > 1800 s). It is well known that whey protein powders show poor wetting behaviour, the material floats on the water surface, which is considered to be the rate-limiting factor particularly for whey proteins (Gaiani et al., 2009). On the other hand, it was observed that whatever the storage condition, the powders presented excellent rehydration capacity with high dispersibility. However, it can be noticed that a small decrease in solubility can be observed for the powders stored in the conditions of set 1, corresponding to the highest temperature.

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

### **3.2.1. Protein lactosylation and aggregation**

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

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

### 3.2.2. Identification of proteins present in aggregates

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

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

#### 4. Discussion

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

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

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

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**

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

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

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

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

## 5. Conclusion

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



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

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

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

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

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## 7. Conflict of interest

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

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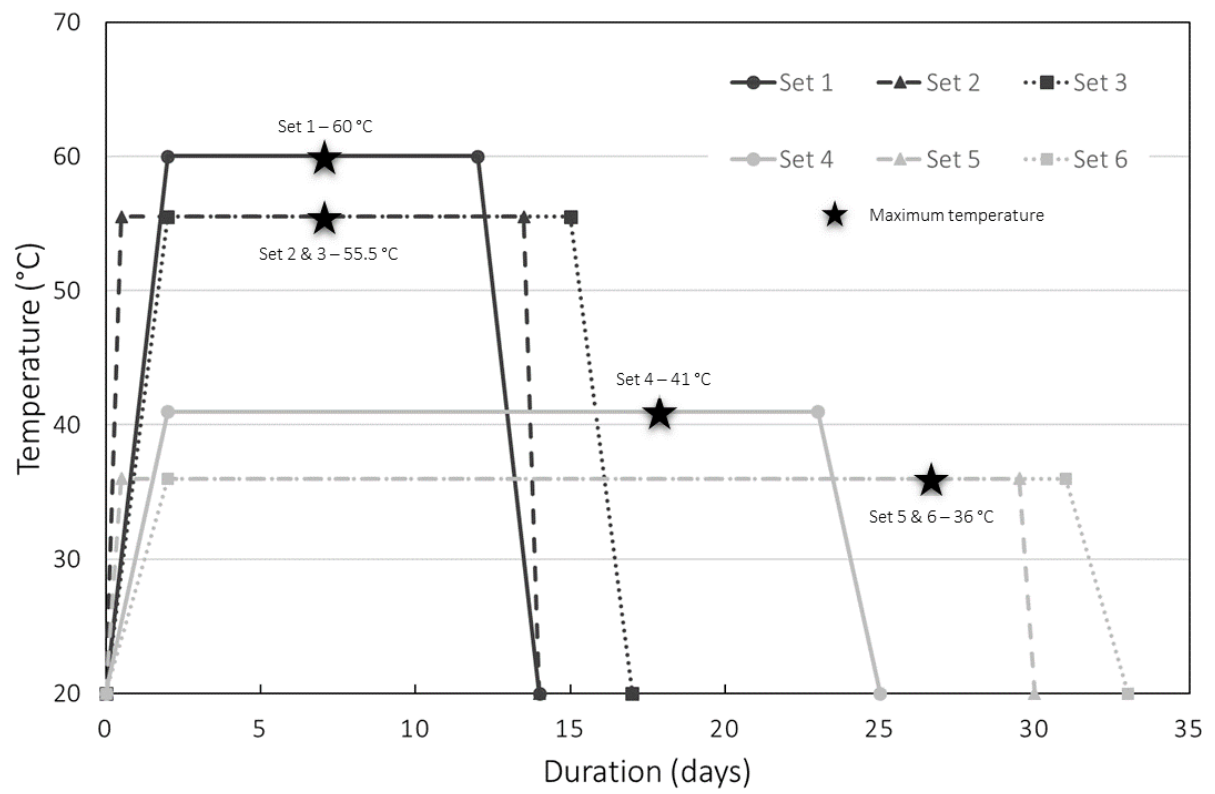
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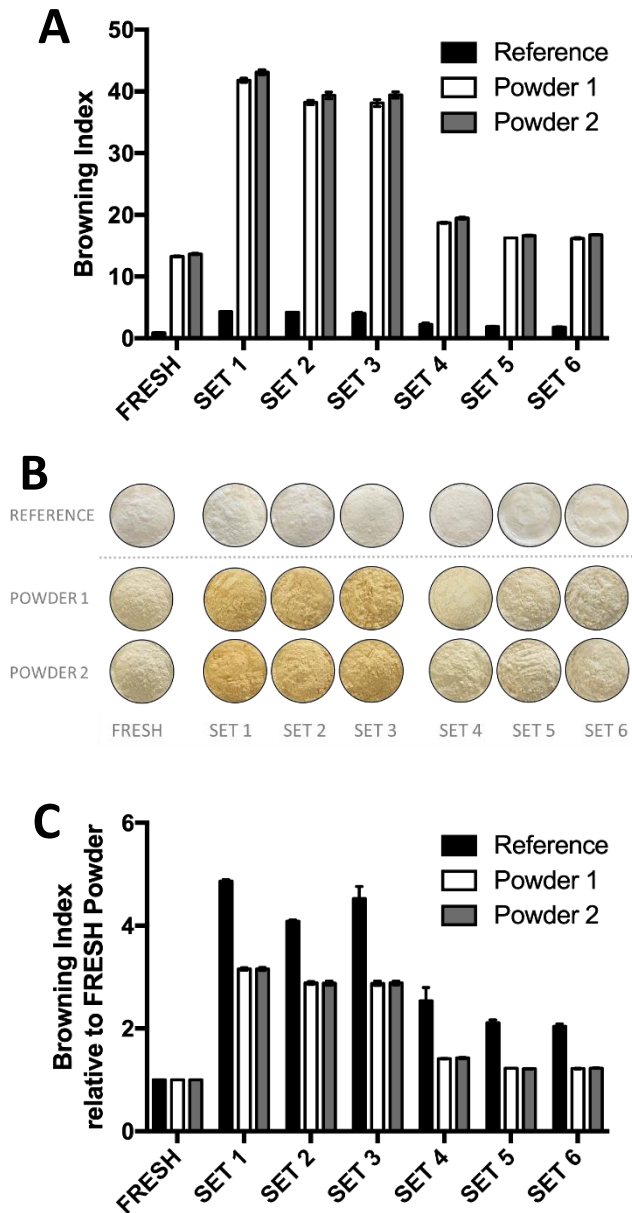
**Table 1:** Chemical composition (n=2) for powders 1, 2 and reference

	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
Total proteins (g.100g <sup>-1</sup> )	79.6 ± 0.1	84.1 ± 1.1	83.9 ± 3.9
Caseins (% of total proteins)	0.9 ± 0.4	5.4 ± 0.3	-
β-LG (A) (% of total proteins)	26.0 ± 0.3	24.7 ± 0.2	31.6 ± 0.1
β-LG (B) (% of total proteins)	48.5 ± 0.2	46.5 ± 0.2	68.4 ± 0.4
α-LA (% of total proteins)	24.6 ± 0.2	23.4 ± 0.1	-

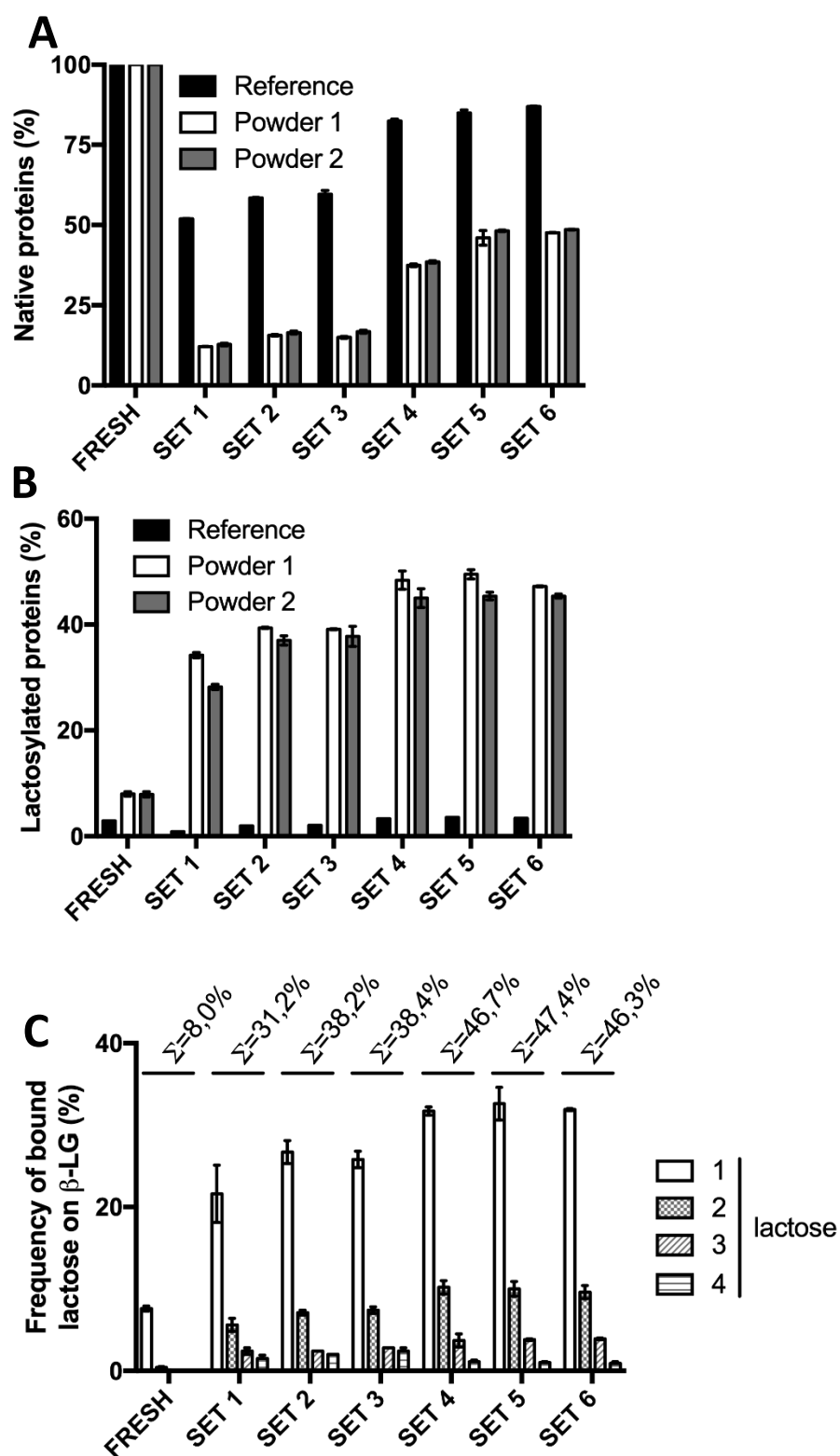
\*Data provided by AFI



**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.

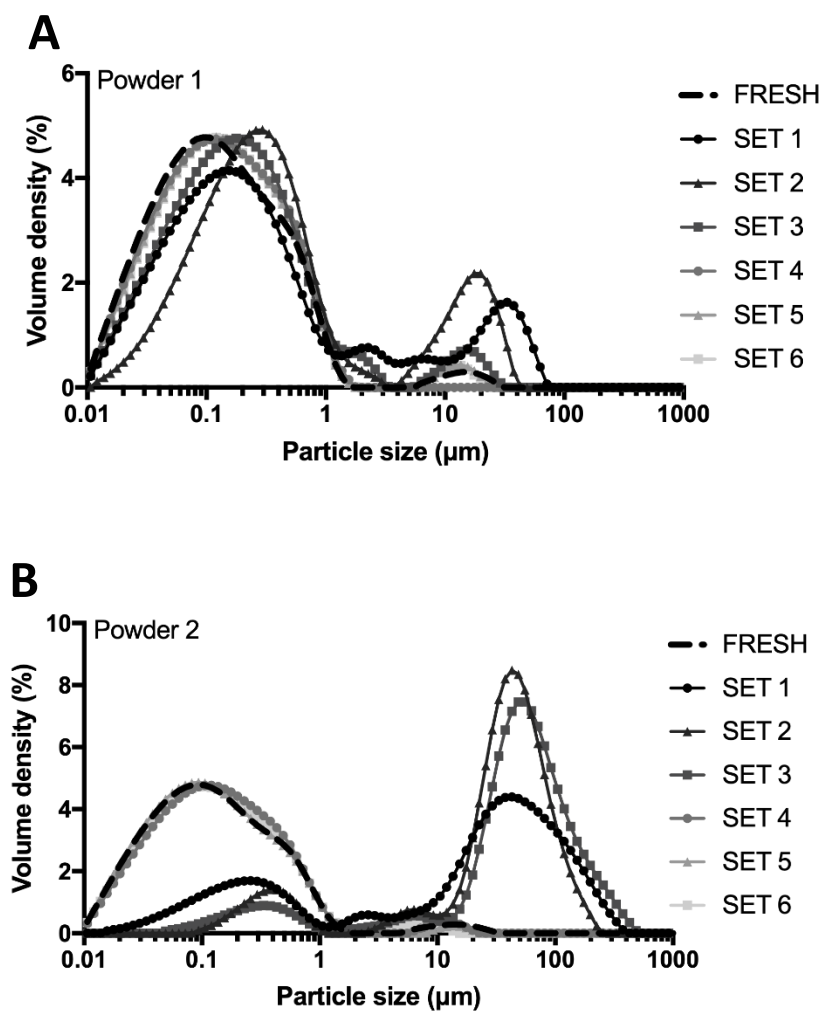
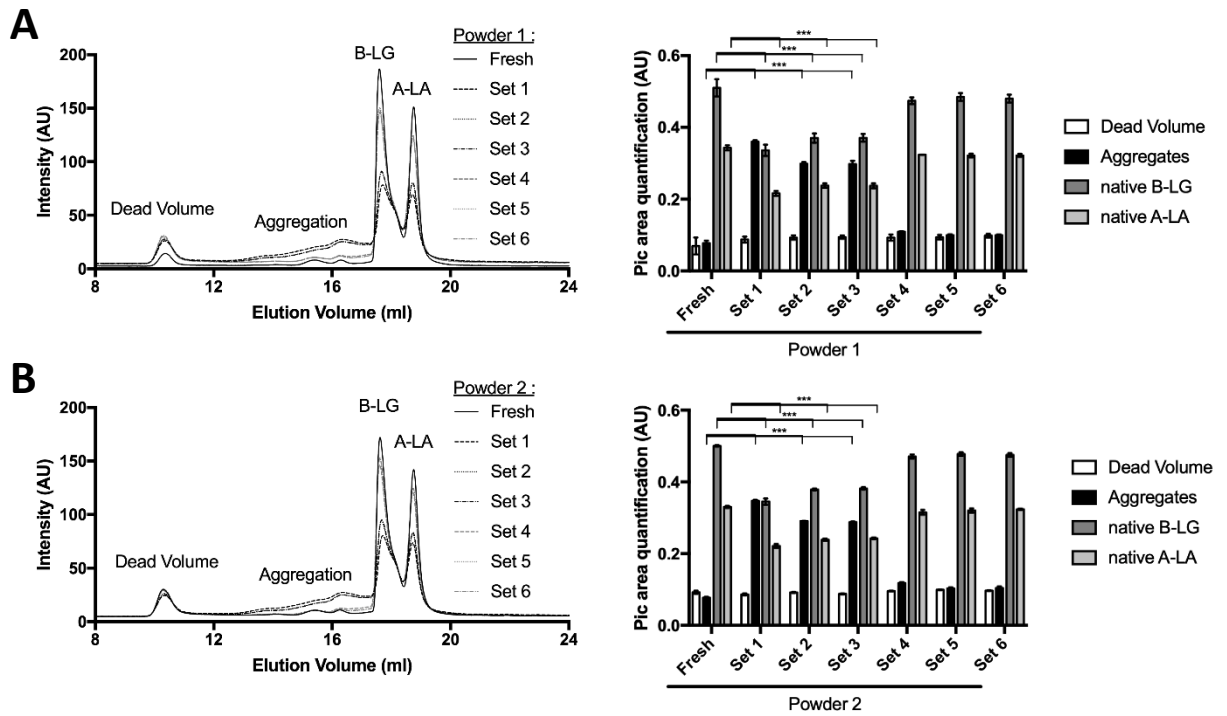
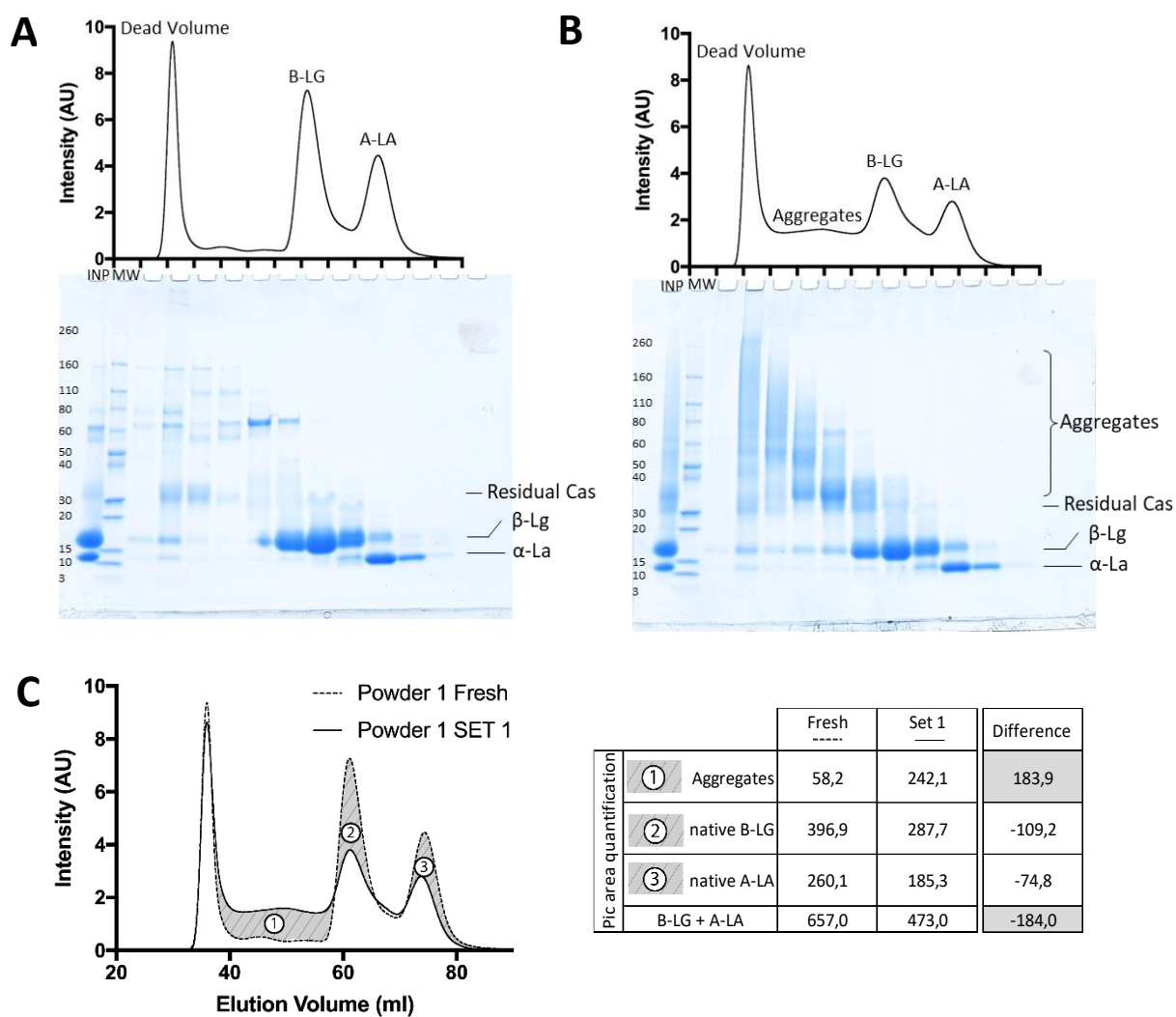


Figure 4: Particle size distribution after powder reconstitution. (A) Powder 1. (B) Powder 2.



**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).