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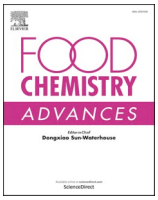

Effects of storage temperature on the solubility of cross-linked micellar casein powders

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ABSTRACT

Micellar casein powders (MCP) are added to food products to enhance their protein content and functional qualities. Enzymatic cross-linking (CL) with microbial transglutaminase (mTGase) is a promising method for improving the functional properties of MCP. Non-cross-linked (N-MCP) and enzymatically cross-linked (C-MCP) at different CL degrees (0, 36, 48, and 58 %), were evaluated to assess the changes in MCP powder solubility after storage at two temperatures (25 and 40 °C) for up to 180 days. The MCP were examined periodically to determine the browning index (BI), the loss of solubility, the variations in the wetting time, and the morphological surface composition evolution with aging. The solubility of both N-MCP and C-MCP decreased similarly with storage, and this phenomenon was enhanced by increasing the severity of the storage (temperature and time). Based on changes in the BI and solubility loss, N-MCP and C-MCP have similar aging kinetics. According to relaxation time analysis, the CL degree reduced the interaction between the powder particles and the solvent, which further diminishes with storage. The results suggest that CL would trigger changes in the MC powder surface, but the evolution of the functional properties is not related to the storage-induced CL formation.

Introduction

Bovine milk main components are water, lactose, fat, proteins (whey protein and caseins), vitamins, and minerals. To facilitate handling, shipping, and storage, milk can be dehydrated by spray drying. Milk's composition can be modified through filtration technologies (microfiltration, ultrafiltration, nanofiltration, and reverse osmosis) to obtain different types of milk powders. Phosphocaseinate, or micellar casein powder (MCP), are a type of high-protein dairy powder obtained after the microfiltration (MF) and spray drying of skim milk. They contain up to 84% of protein, primarily caseins in a micellar form (Hammam et al., 2021). The MCP is widely used in the industry to standardize the protein content and to provide texture to food products.

Fast and complete rehydration of the milk and MCP is required for food applications. The MCP is known for its poor solubility, which increases with severe aging conditions (temperature, humidity, and storage time) (Anema, Pinder, Hunter & Hemar, 2006). From literature, it has been suggested that the loss of protein solubility can be caused and observed by different factors: covalent inter-micellar cross-linking formation (Le, Holland, Bhandari, Alewood & Deeth, 2013), phospholipids migration from the core to the surface of the milk powders (Gaiani et al., 2007), changes in the surface structure of the milk powders, notably the formation of a caseins rough crust zones and hollow zones on the powder surface (Burgain, Scher, Petit, Francius, Gaiani, 2016), conformational modifications (loss of α-helix content) (Nasser et al., 2018) and the development of casein protein-protein hydrophobic interactions (Anema et al., 2006; Fyfe et al., 2011; Haque, Bhandari, Gidley, Deeth & Whittaker, 2011; Havea, 2006).

The microbial transglutaminase (mTGase) is an enzyme that catalyzes the formation of covalent cross-linking by catalyzing acyl transfer reactions between glutamine and the γ-carboxamide group of peptide or protein-bound glutamine (acyl donor) and primary amines (acyl acceptor) including the ε-amino group of lysine residues (Jaros et al., 2006). The products of the cross-linking reaction (dimers, trimers, and

Abbreviations: BI, Browning Index; ΔDP, Degree of polymerization; CL, Cross-linking; C-MCP, Cross-linked Micellar Casein Powders; MCP, Micellar casein powders; MCC, Micellar casein concentrates; mTGase, Microbial transglutaminase; N-MCP, Non-cross-linked Micellar Casein Powders.

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oligomers) can be quantified by Size Exclusion Chromatography (SEC). The relationship between the peak area of the reaction products and the sample's overall peak area gives the degree of polymerization (DP), which can be viewed as a quantitative indicator of the cross-linking reaction effectiveness (Velazquez-Dominguez et al., 2023).

Most studies on enzymatic modification of casein micelle have been performed on liquid systems where no drying is performed. In these systems, it has been reported that the addition of mTGase increases the apparent viscosity of the yogurt and increases the stiffness of the gels (Gharibzahedi & Chronakis, 2018; Gharibzahedi et al., 2018). As for studies dealing with the cross-linking of casein micelles followed by a drying operation, an increase in stiffness and water holding capacity in heat-induced gels produced with cross-linked milk powders (Imm, Lian, Lee, 2000) and an increase in apparent viscosity of stirred yogurt while reducing serum loss (syneresis) (Guyot & Kulozik, 2011) have been reported.

In addition to the functional properties of cross-linked milk powders, some authors have studied the structure and flow properties of these powders (Er, Sert & Mercan, 2019; Romeih, Albadarin, Olaleye & Walker, 2021). However, to our knowledge and despite the scientific and industrial pertinence, no study has so far correlated the structural changes with the techno functionalities such as rehydration capacity of cross-linked MCP through storage.

The objective of this study is to better understand and quantify effect of storage time, temperature, and how crosslinking intervenes in the properties of MCPs. To do so, we quantified the changes on some techno-functional properties (the browning index, solubility, wettability time) of enzymatically cross-linked micellar casein powders (ΔDP= 0, 36, 48, and 58%) that were stored for up to 180 days under controlled storage temperatures (40 and 25 °C) with a different relative humidity of 10.4 and 21%, respectively.

Materials and methods

Cross-linked micellar casein concentrate powders (MCP) manufacture: physicochemical analysis and process parameters

Micellar casein concentrate (MCC) was obtained through ultrafiltration and microfiltration of skimmed milk from Ingredia Dairy Experts (Arras, France). Four independent batches of MCC were added with microbial transglutaminase (Activa YG, Ajinomoto, batch 202.103.021, declared enzymatic activity of 109 EU) in the following conditions: MCC-0%: without added enzyme, MCC-36%: incubated with 1 $U.g^{-1}$ protein for 2 h, MCC-48 %: incubated with 1 $U.g^{-1}$ protein for 8 h and MCC-58%: incubated with 3 $U.g^{-1}$ protein for 8 h. These conditions of incubation and enzyme concentration were chosen due to previous experiments that showed that these conditions provided a different range of polymerization degrees (Velazquez-Dominguez et. al., 2023). After the incubation, the enzyme was inactivated by thermal treatment at 80 °C for 1 min. The inactivated samples were placed again at 40 °C (the optimal enzyme temperature) for at least 2 h. The samples were considered as inactivated since the degree of polymerization did not continue to increase (determined by size exclusion chromatography). After the inactivation step, the cross-linked MCC was spray-dried in a pilot workshop (GEA, Niro Atomizer, St Quentin en Yvelines, France) at Ingredia facilities (Arras, France). The inlet temperature during the spray-drying was 178 °C, and the outlet temperature was 80 °C. Micellar casein powders (MCP) were packed into individual 180 g pack-lab aluminum bags that were hermetically sealed. The powders were stored for 7, 30, 60, 90 and 180 days at 40 °C and 25 °C with constant a relative humidity of 10.4 and 21.0%, respectively.

Quantification of the polymerization degree

The degree of protein polymerization was determined by using a size exclusion chromatography method (SEC), according to Guyot and Kulozik (2011). An ÄKTA FPLC system provided a P900 pump, a variable wavelength UV detector, and a Superdex 200 10/300 GL gel filtration column (Amersham Biosciences, Freiburg, Germany) were used. The column was eluted at room temperature at a flow rate of 0.75 $mL.min^{-1}$ with elution buffer composed of 6 M urea, 0.1 M sodium chloride, and 0.1 M sodium phosphate. 100 µL of the reconstituted powders at 2.7% w/w of protein were injected by triplicate. Data evaluation and peak integration were done using the Unicorn 4.11 software (Amersham Biosciences, Freiburg, Germany). The degree of enzyme-induced protein polymerization, degree of polymerization (DP), was defined as the number of cross-linked caseins related to the total amount of caseins in the sample (Bönisch, Lauber & Kulozic, 2004) DP was calculated according to Eq. (1):

$$DP = \frac{Acl}{At} x\ 100 = \frac{Ap}{Apm} x\ 100$$

$$\Delta DP = DP\ mTGase - DP\ without\ mTGase \quad (1)$$

Where *Acl is* the area of cross-linked caseins, *At* is area of total caseins *Ap* is area of polymers (oligomers, trimers and dimers), and *Apm* is area of oligomers, trimers, dimers and monomers.

The cross-linking or polymerization degree of the MCP results is depicted in **Table SI-1**. ΔDP increases when the enzyme concentration and/or the incubation time increases and they are in agreement with previous cross-linking degree estimated values (Velazquez-Dominguez et al., 2023). In the following sections, we will refer to the powders according to their ΔDP as MCP-0%, MCP-36%, MCP-48%, and MCP-58%.

Physico-chemical analysis of the Micellar casein powders

The methods for the determination of the physicochemical properties including moisture content, total nitrogenous matter, non-proteinic nitrogen, non-casein nitrogen, lactose, lipids, ash, soluble and total calcium and particle size are specified in the supplementary material (SI-1).

Browning index determination of micellar casein powders

The MCP color variation evaluation through storage was carried out with a R-3L00 Minolta colorimeter (Konica Minolta, Osaka, Japan). Before the measurements, the instrument was calibrated with a white reference tile (L*=96.03, a*=4.71, b*=7.24). The L* (0=black, 100=white), a* (where respectively negative and positive values indicate green and red) and b* (where respectively negative and positive values indicate blue and yellow) color coordinates were determined according to the CIELAB coordinate color space system. The MCP were homogenously distributed on a Petri dish and color measurement was carried out on three different points on the surface of the Petri dish receptacle. The browning index (BI), used to assess the intensity of the brown color of the samples, was calculated from Eq. (2) where the L*a*b* values corresponds to the CIELAB space, where L* corresponds to the lightness, a* corresponds to the green/red values and b* to the blue/yellow values (Nasser, Moreau, Jeantet, Hédoux & Delaplace, 2017)

$$BI = \frac{[100\ (x - 0.31)]}{0.17} \quad (2)$$

With

$$x = \frac{a^* + 1.750\ x\ L^*}{5.645\ x\ L^* + a^* - 3.012\ x\ b^*}$$

Determination of powders solubility

Determination of the amount of soluble material in the supernatant

An aqueous solution of the MCP were dispersed in milli-Q water at

5% (w/w) at 20 °C for 1 h under stirring. Later, 50 mL of the MCP dispersions were centrifuged using a Sigma 6K15 refrigerated centrifuge (Sigma, Labozentrifugen GmbH, Osterode am Harz, Germany) at 700 × *g* at 20 °C for 20 min. After centrifugation, the supernatant of the dispersions was carefully collected and placed in pre-weighed moisture dishes. The moisture dishes were then placed in an oven at 105 °C for 24 h. After this time, the moisture dishes were placed in a desiccator filled with dry silica gel to avoid condensation. The moisture dishes were then weighed. The percentage of soluble material (σ) was calculated with Eq. (3) (Anema et al., 2006)

$$\sigma = \frac{Wd}{Ws}\ x\ 100 \tag{3}$$

Where *Wd* is the weight of dry material and *Ws* is the Weight of solution.

The σ values of aged MCP were compared to that of the reference MCP (value of MCP before storage), according to Eq. (4):

$$\sigma_i = \frac{\sigma_i}{\sigma_r} \tag{4}$$

Where σ_i is the aged at storage day "i" and σ_r is the reference. One should notice that the solubility determined by this method comprises the sum of all the supernatant constituents, which are composed mainly of highly soluble material (soluble calcium, lactose and ash), which varies within the MCP-38% and 58% (Table 1). For that reason, we decided to evaluate the protein solubility in the supernatant after 1 h of rehydration by high-performance liquid chromatography (HPLC). We assess the solubility of the MC powders after 1 h of rehydration since it was a recommendation from the casein powder supplier. It should be noted that the reconstitution time of milk powders depends on factors such as temperature and chemical composition of the milk powders, and it can take up to more than 24 h to achieve complete rehydration (Wu et al., 2022).

Quantification of proteins concentration in the supernatant by High-Performance Liquid Chromatography (HPLC)

Aqueous solutions of 5% (w/w) MC powder were prepared in the same way as in Section 2.5.1. The content of soluble caseins was then determined by High-Performance Liquid Chromatography (HPLC) system (Alliance, HPLC System, Waters, USA). The chromatographic system consists of a separation module integrated solvent and sample management function (e2695 separation module), a column heater/cooler system, an ACE 5 C4–300 column (length 250 mm, Ø 3 mm, and 5 μm particle size) (ACE Ltd., Aberdeen, UK), a UV–VIS spectrophotometer (2898 PDA Detector) and acquisition software (Empower Software). The supernatants were diluted with urea (6 M) and filtered through a 0.2 μm cellulose filter (Minisart, RC, Sartorius, Germany) before injecting 20 μL into the HPLC system. Two mobile phases were used: solvent A (H2O/0.1% trifluoroacetic acid) and solvent B (acetonitrile/0.1% TFA) (HPLC grade, Thermo Fisher Scientific, France). The separation was performed at a flow rate of 1 mL.min^{-1}, 40 °C, and a detection wavelength of 215 nm with gradient elution. Calibration curves were performed using pure casein standards at 0.25, 0.5, 1, and 2 g/L.

Table 1
Physico-chemical analysis of the non-cross-linked (0 %) and cross-linked MCP at different polymerization or cross-linking degree ΔDP (36, 48, 58%).

	MCP-0 %	MCP-36 %	MCP-48 %	MCP-58 %
pH	7.04 ± 0.05 ^{a}	7.04 ± 0.05 ^{a}	6.97 ± 0.07 ^{a}	6.77 ± 0.09 ^{b}
Moisture content (%)	5.76 ± 0.19 ^{a}	5.73 ± 0.32 ^{a}	5.33 ± 0.18 ^{ab}	5.04 ± 0.29 ^{b}
Total nitrogenous matter content (%)	81.84 ± 0.11 ^{a}	82.85 ± 0.11 ^{b}	81.16 ± 0.38 ^{c}	80.38 ± 0.41 ^{d}
Non proteinic nitrogen (NPN) (%)	0.74 ± 0.11 ^{c}	0.92 ± 0.12 ^{c}	1.25 ± 0.10 ^{b}	2.48 ± 0.09 ^{a}
Non casein nitrogen (NCN) (%)	2.84 ± 0.10 ^{b}	2.82 ± 0.06 ^{b}	2.90 ± 0.12 ^{b}	4.92 ± 0.20 ^{a}
Lactose (%)	3.40 ± 0.25 ^{b}	3.18 ± 0.51 ^{b}	4.06 ± 0.31 ^{ab}	4.49 ± 0.27 ^{a}
Lipids (%)	0.95 ± 0.1 ^{b}	1.30 ± 0.26 ^{ab}	1.56 ± 0.3 ^{ab}	1.72 ± 0.27 ^{a}
Ash (%)	8.29 ± 0.04 ^{a}	7.46 ± 0.08 ^{b}	8.21 ± 0.02 ^{a}	8.22 ± 0.08 ^{a}
Total calcium (ppm)	26106 ± 442 ^{a}	23803 ± 157 ^{b}	25643 ± 317 ^{a}	26006 ± 255 ^{a}
Soluble calcium (ppm)	273 ± 18 ^{c}	381 ± 44 ^{b}	442 ± 37 ^{b}	754 ± 20 ^{a}
D [4,3] (μm)	15.0 ± 0.1 ^{c}	29.0 ± 0.1 ^{a}	24.2 ± 0.1 ^{b}	26.0 ± 0.3 ^{ab}
D [3,2] (μm)	3.2 ± 0.0 ^{c}	5.4 ± 0.0 ^{a}	5.0 ± 0.0 ^{b}	5.1 ± 0.8 ^{ab}

Results are given as average confidence interval (α =0.05); different letters (by row) indicate significant differences with a 95 % confidence level.

Wetting time determination: relaxation time test

The relaxation time was obtained using an non-commercial ultrasound (US) test developed by Richard et al. (2012), which measures the kinetics of the occluded and interstitial air release from the MCP particles after immersion in water. It reflects the resistance water encounters to diffuse toward the core of the particles. For this test, MC powder was rehydrated at 0.2% w/w in 1 L of distilled water in a beaker, using a magnetic stirrer (SI Analytics GmBH, Mainz, Germany) at 450 rpm. The water temperature was set at 30 °C and controlled throughout the experiment. The ultra-sound probe was immersed in the beaker, and the powder was added once the sound wave amplitude baseline was stable. The release of air entrapped in particles is responsible for the extinction of the US signal (Richard et. al., 2012). As water progressively replaced the internal air of the particles, the air release flow rate decreased, and the signal moved toward an asymptotic plateau. The relaxation time was defined as the lapse of time from signal extinction upon powder incorporation to its recovery to 90% of the initial signal value.

Determination of protein interactions in the insolubles after centrifugation by SDS-PAGE

Four MCP (ΔDP = 0, 36, 48 and 58 %) stored at 40 °C for 180 days and four MCP non-stored MCP were rehydrated under constant stirring (300 rpm) at 50 °C for 36 h. The MCP dispersions were centrifuged at 700 *g* for 20 min at 20 °C. After centrifugation, the pellet and the supernatant were separated. The pellet of the 8 MCP dispersions was weighted and added with a solution containing 0.1 M EDTA, 8 M urea, 0.2% SDS, 0.05% β-mercaptoethanol, 7% glycerol, and 0.01% bromophenol blue and heated at 80 °C for 5 minutes. The separation gel was composed of 15% acrylamide (2.6% Bis), 0.1% SDS in 1.5 M of Tris-HCL buffer (pH 8.8). The stacking gel was composed of 4% acrylamide, and 0.1% SDS in 0.5 M Tris/HCl buffer (pH 6.8). 10 μL of the samples were loaded into the separation gel, and separation was performed using a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) at 100 V and 0.5 mA. Later, gels were incubated for one h in the staining solution containing 0.15% Coomassie Blue, ethanol (45%), and acetic acid (7.5%). After that, the gels were destained two times in distilled water for 24 h. The gels were scanned using a Perfection V800 Photo scanner (EPSON, France).

Analysis of protein and lipid intensity in the micellar casein powder surface by Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

ToF-SIMS was carried out to obtain information about the composition of the surface of the powder (1–3 nm depth) (Nasser et al., 2017). Lipid and amino acids intensity on the MCP surface were performed using a ToF-SIMS spectrometer (IONTOF GmbH, Germany) equipped with a bismuth liquid metal ion gun. The compacted MCP were analyzed with a pulsed Bi_3^+ primary ion beam (25 KeV, 0.25 pA). The intensity of the lipid and amino acids at the extreme surface was obtained from 3

scan surface areas (500 μm x 500 μm). The protein intensity was monitored by the presence of mass spectrum area of the primary amino acids of the MCP: Glycine (CH_4N^+at $m/z = 30{,}03$), Alanine ($C_2H_6N^+$ at $m/z = 44{,}05$), Serine ($C_2H_6NO^+$ at $m/z = 60{,}05$), Proline ($C_4H_6N^+$ at $m/z = 68{,}04$), Valine ($C_4H_{10}N^+$ at $m/z = 72{,}08$), Threonine ($C_3H_8NO^+$ at $m/z = 74{,}06$), Cysteine ($C_2H_6SN^+$ at $m/z = 76{,}02$), Leucine ($C_5H_{12}N^+$ at $m/z = 86{,}1$), Aspartic acid, asparagine ($C_3H_6NO_2^+$ at $m/z = 88{,}03$), glutamic acid ($C_4H_8NO_2^+$ at $m/z = 102{,}06$), and Histidine ($C_5H_8N_3^+$ at $m/z = 110{,}07$) (Tyler, Bruening, Rangaranjan & Arlinghaus et al., 2011). The presence of lipids was monitored by the secondary ion signal of phosphocholine ($C_5H_{15}PNO_4^+$ at $m/z = 184{,}09$) since phosphocholine is one of the two major milk phospholipids with low solubility (Nasser et al., 2017). The intensity of the protein and lipid on the surface was calculated by Eq. (5) and Eq. (6), respectively.

$$\text{Intensity of protein at the extreme surface} = \frac{\sum_p = \text{area of mass spectrum of protein fraction}}{\sum_a = \text{area of mass spectrum of protein fraction} + \text{area mass spectrum of the lipid fraction}} \quad (5)$$

$$\text{Intensity of lipid at the extreme surface} = \frac{\sum_l = \text{area of mass spectrum of lipid fraction}}{\sum_a = \text{area of mass spectrum of protein fraction} + \text{area mass spectrum of the lipid fraction}} \quad (6)$$

Powders surface observation

Scanning electron microscopy (SEM)

A high-resolution field-emission scanning electron microscopy (SEM) type JEOL JSM-7800FLV supplied with a hot (Schottky) electron gun (JEOL Ltd., Tokyo, Japan) with a scanning voltage of 5 kV was used to investigate the surface morphology and structure of MCP in a dried state. Briefly, to prepare the samples, a thin layer of the MCP was sprinkled and fixed on specimen stubs with double-sided adhesive tape (Agar Scientific, Oxford).

Results and discussion

Physico-Chemical analysis results

Table 1 provides a description of the powders' chemical analysis. The utilization of four distinct MCC batches can account for the variations in the lactose and lipid content of the MCP. For MCP-58%, there was a small but statistically significant drop in pH. The pH drop can be attributed to the longer (8 h) incubation period with mTGase at 40 °C prior to the spray drying process. It is in fact known that heating milk to high temperatures and/or for extended periods of time causes the precipitation of calcium phosphate, which causes heat-induced acidity of milk (O'Connell & Fox, 2003b). As the degree of cross-linking increased, the moisture content in the MCP dropped, most likely because there was more water in the cross-linked MCC available to move from the droplet to the drying air (Shuck, 2014). The resistance of the cross-linked casein micelles against calcium heat-induced precipitation is most likely what causes the rise in soluble calcium when cross-linking is increased (Huppertz, 2014). It is well known that heating methods (such as pasteurization or ultra-high-temperature pasteurization) cause the CCP's solubility to decrease (Nogueira et al., 2023). Because the micellar structure was reinforced through cross-linking, more casein micelles were retained during the spray-drying process, which resulted in higher soluble calcium concentrations due to the creation of covalent cross-linking. This could have restricted the quantity of calcium phosphate lost during the process. Moreover, non-casein and non-proteinic nitrogen display some increase as a function of the DP. This is expected as a side effect of the cross-linking since the chemical process liberates the organic nitrogen group of lysine.

Color measurement and browning index (BI) determination

The Maillard reaction is a chemical reaction related to changes in color, mostly in food products. The final part of the Maillard reaction involves the degradation of Amadori compounds, which start to polymerize and generate high molecular weight products with brown pigmentation. The formation of brown pigments is influenced by the ratio of sugars and amino acids within the substrate (Aalaei, Rayner & Sjöholm, 2019). **Figure SI-1** shows that the cross-linked powders are notably browner than the MCP-0% powder.

The color differences between the produced MC powders, expressed as Browning Index (BI), are illustrated in Fig. 1**a**. Since BI of the powders stored for 7 days at 40 °C (as depicted in Fig. 1) were minimal, we assumed that the variations of BI for 7 days at only 25 °C would be below the sensitivity of our detection method. The BI of the powders before storage is significantly higher for cross-linked MCP than for non-cross-linked MCP (Fig. 1**a**). The BI difference between the non-cross-linked and cross-linked MCP might be explained by the addition of a mTGase preparation mixture that possessed a yellowish color. The BI index difference between the cross-linked MCP is likely cause by the different enzyme concentrations used to manufacture the MCP. Higher enzyme concentrations were used for the fabrication of MCP-58% (3 $U.g^{-1}$ protein) than for MCP-36 and MCP-48% (1 $U.g^{-1}$ protein).

The evolution of the browning index (BI) as a function of storage time (Fig. 1**b**) shows that BI is strongly influenced by the storage time and storage temperature: BI increases faster when storage temperature increases from 25 to 40 °C. The BI for all the cross-linked powders stored at 40 °C for 180 days is notably higher than for the MCP-0%, but one should notice that the BI evolution of cross-linked MC powders follows the same trend (same slope) as CM-0%. If the BI are normalized and their slopes compared (Fig. 1**c**), one can notice that MCP-36 % depicts a slightly higher BI compared to the other MC powders. When the storage temperature is 40 °C, it takes about 180 days to double the initial BI value of all the MCP. At 25 °C, the initial BI value remained practically the same after 180 days of storage. Overall, the effect of the mTGase addition to the micellar casein retentates before the spray-drying operation affected the initial BI value of the MCP. Still, the BI evolution through storage is not affected by the cross-linking of the MCP.

Powder surface observation

The MCP-0, 48, and 58% were observed by SEM before and after storage at $t_s = 90$ and 180 days. From **Figure SI-2**, one can observe notorious morphological differences between the non-cross-linked and cross-linked MCP. Cross-linked MCP particle size is bigger than non-cross-linked MCP. The D [4,3] and the D [3,2] values (Table 1), that in particle size distribution measurements represent the powder particles mean volume diameter and the surface mean diameter, respectively, are significantly bigger for cross-linked MCP than MCP-0%. These differences are probably related to differences in the evaporation rate and

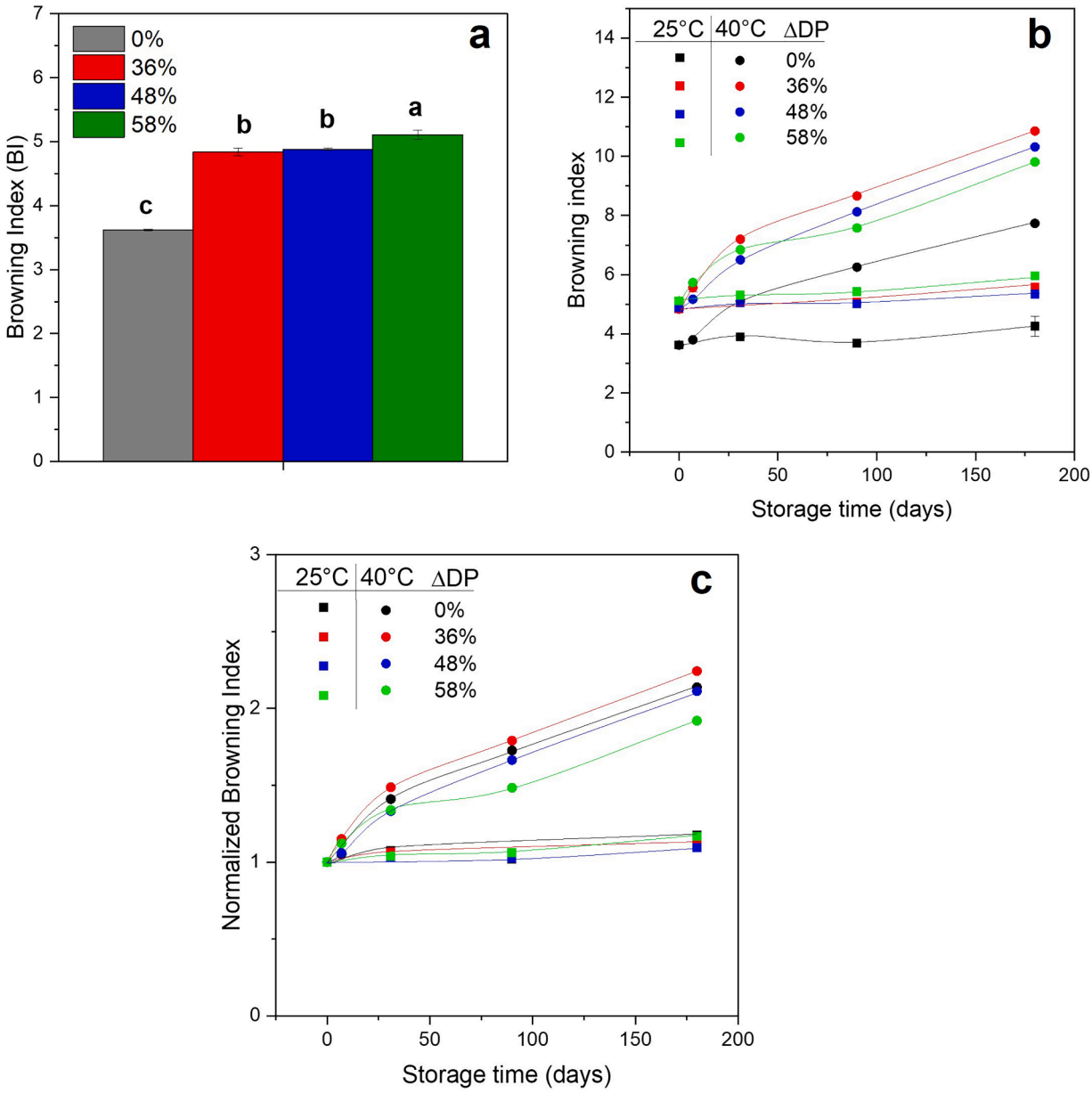

Fig. 1. Browning index (BI) of MCP 0, 36, 48, and 58 % a) before storage b) as a function of storage time at different temperatures (40 and 25 °C) and c) Normalized BI as a function of storage time. The lines used in are only a guide to the eye.

water transfer of non-cross-linked and cross-linked micellar casein retentates during the spray-drying process. The formation of wrinkled or spherical powder particles depending on the conformation (globular or sponge-like) of the protein indicate that the protein characteristics plays an important role in the protein and lipid layer formation during the spray-drying operation (Sadek et al., 2015; Yu et al., 2021). It is likely that formation of casein polymers during the cross-linking of the micellar casein retentate have caused changes on the water transfer during the spray-drying, leading to changes in the size and shape of the MCP particles.

Effect of storage conditions on lipid and protein mobility towards the MCP surface

Fig. 2**a** and 2**b** depict the intensity of the protein and lipids at the micellar casein powder detected by ToF-SIMS, respectively. Before storage, the intensity of the lipid at the surface of MCP-0% was 0.016 A.U., and protein intensity was 0.983 A.U. Before and after storage, MCP-48 and MCP-58% shown a higher protein intensity than MCP-0%. At t_s=180 days, a significant ($p > 0.05$) increase was observed in the lipid intensity at the surface (0.023 A.U.) of MCP-0% whereas no lipid intensity evolution was detected for MCP-48 and 58%.

The higher level of detected protein in the outer surface of cross-linked MCP is in line with the assumption of different characteristics of the external protein layer formed in non-cross-linked and cross-linked micellar casein retentates during the spray-drying process. Moreover, from Fig. 2**b**, one can notice that the increase of the lipid migration towards the surface is diminished by cross-linking. MCP-0% depicts a significant increase of the lipid after 90 and 180 days of storage. MCP-48% has a significant increase of the lipid content after 180 days and MCP-58% does have any increase in the lipid content after storage. It is thus feasible to suggest that a "dense" protein layer is formed during the spray-drying of cross-linked micellar casein retentates. This layer does not evolute during the storage, but it could play a role in diminishing the migration of lipids towards the MCP particles.

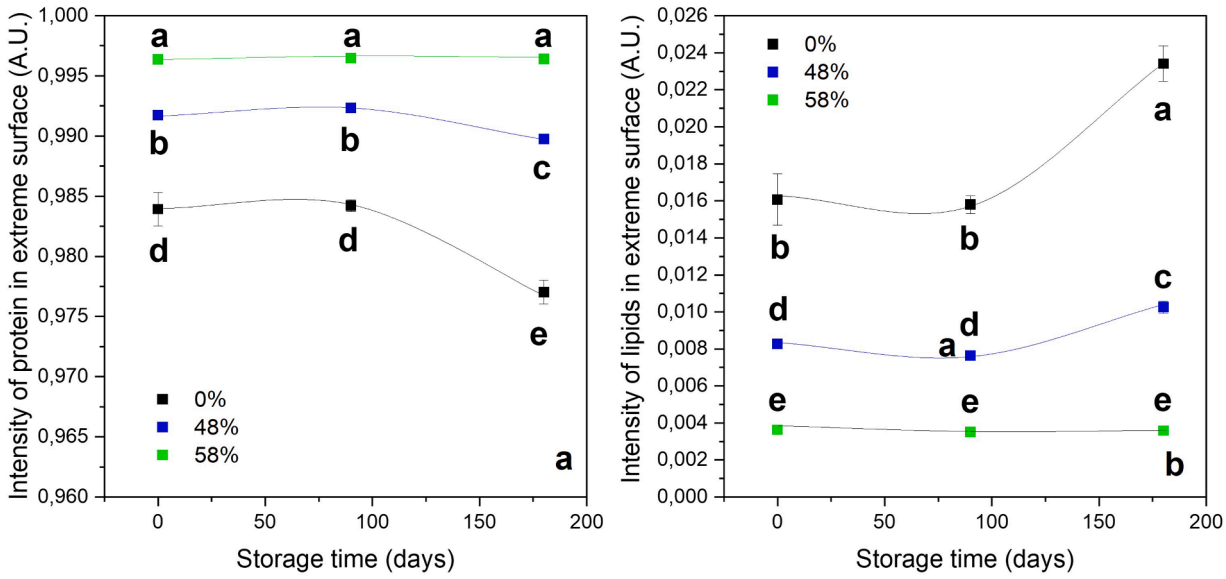

Fig. 2. Intensities of the a) amino acids fraction and b) lipid fraction at the extreme surface of the non-cross-linked (0 %) and cross-linked (48 and 58 %) MCP as a function of the storage time (storage temperature = 40 °C). The lines used in are only a guide to the eye.

Wetting time determination

To evaluate the interaction between the MCP particles and the solvent the wetting time was determined. The rehydration of milk powders, is a process that involves several steps: wettability, swellability, sinkability, dispersibility, and solubility (Shuck, 2014). Given that spray drying depletes caseins of their natural medium (water) and replaces it with a relatively "hydrophobic" medium (air), the equilibrium of interaction among all the hydrophobic and polar components in the sample undergoes a shift. During storage, time and temperature will provoke a slow evolution of the molecules toward a new equilibrium (Fialho et. al., 2019; Nasser et al., 2018b). In this scenario, small hydrophobic molecules as the lipids may slowly migrate during time (Nasser et. al., 2017). Besides temperature, this migration will be a function of the overall dynamics of the medium, that in this case is mainly composed of caseins. Thus, the difference in lipid migration between the casein cross-linked and non-crosslinked since cross-linking reveals a difference in the dynamics of the casein medium induced by cross-linking.

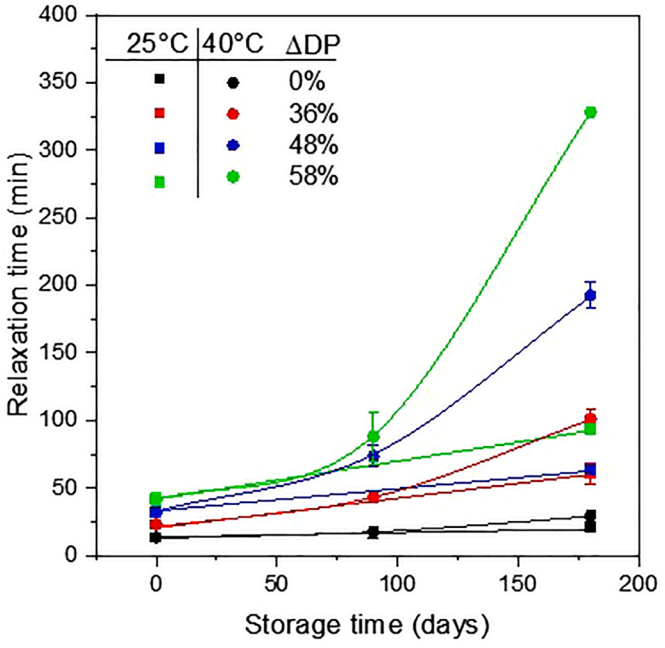

Fig. 3. Relaxation time of non-cross-linked (0 %) and cross-linked MCP at different polymerization or cross-linking degree ΔDP (36, 48, 58 %) at two storage temperatures (25° and 40 °C) as a function of the storage time. The lines used in are only a guide to the eye.

The first step of the rehydration process, the wettability, indicates the capacity of water transfer from the surface to the core of the MCP (Nasser et al., 2017; Richard et al., 2012). The trapped air in the powder particles (occluded air) and the air between the individual powder particle (interstitial air) can be detected by the relaxation time, measured thought the ultrasound test (Augustin, Clarke & Chraven, 2003; Richard et al., 2012), this test can provide information of the interaction between the solvent and the MCP powder separation, at a large scale, during the first steps of the rehydration process.

Fig. 3 shows that the relaxation time increases when the powders' incubation temperature increases from 25 °C to 40 °C. Besides, the relaxation time increases with the cross-linking degree and with the increase of the storage time (from 0 to 180 days). The cross-linking of MCP has been reported as the main driver of decreasing the solubility of the high-protein powders during storage (Schokker et al., 2011). The data presented in Fig. 3 shows that cross-linking affect the evolution of the wetting time through storage. The increase of the wetting time for longer times at more severe conditions can be explained by the interaction and the consequent compaction of micelles and a formation of a casein micelle "patches" at the particle surface, slowing the water penetration (Burgain et al., 2016; Mimouni, Deeth, Whittaker, Gidley & Bhandari, 2010; Schokker et al., 2011) and by an increased "stickiness" between the MCP particles during storage. Larger variations of the wetting time through storage of cross-linked MCP compared to MCP-0 % indicate that the mechanism leading to an increase in the wetting time is triggered by the initial cross-linking degree of the MCP.

Protein and lipid migration results (Fig. 2**a** and 2**b**) suggest little changes related the protein surface occur during storage. The lack of an increase of the protein and lipid intensity through storage of cross-linked MCP and yet, the increase in the wetting time (Fig. 3) may indicate that the water transfer capacity towards MCP agglomerates and individual particle powders of cross-linked MCP takes more time due to their increase resistance to be detached. The exact mechanism of MCP insolubility is not fully established but it seems feasible to suggest that the development of protein-protein hydrophobic interactions triggered by

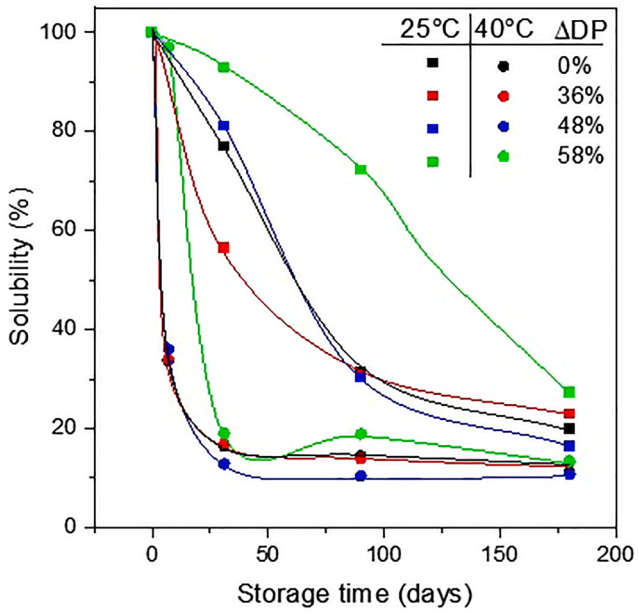

Fig. 4. Change in solubility as a function of the storage time of non-cross-linked (0 %) and cross-linked MCP at cross-linking degree ΔDP (36, 48, 58 %). The lines used in are only a guide to the eye.

the "proximity" between cross-linked casein micelles. This type of protein-protein hydrophobic interactions has been previously described to appear during the storage of high-protein powders (Anema et al., 2006; Havea, 2006)

Powder solubility determination

Determination of the soluble material in the supernatant

The solubility of MCP at different temperatures for 180 days is shown in Fig. 4. The lines used in Fig. 4 are only a guide for eye to help the reader to follow the overall evolution of solubility through storage which reveals to be highly dependent on the storage temperature. Higher storage temperatures resulted in a reduction of MCP's solubility, which was consistent with other research on the storage conditions of high-protein powders (Anema et al., 2006; Le et al., 2011; Nasser, 2017). At 40 °C, the solubility drastically decreases after 7 days of storage. Then, after 30 days of storage at this temperature, a plateau was reached, and the solubility became constant at around 20% of the initial value. At 25 °C, the kinetics of the solubility-loss upon aging is slower than at 40 °C. At 25 °C, the solubility loss decreases with a higher extent of cross-linking (MCP-58 is more soluble than MCP-48 and MCP-36%) whereas MCP-0% has a similar solubility loss as MCP-48%. These results suggest that there is no linear relationship between the loss of solubility and the covalent cross-linking degree, as previous works have suggested (Anema et al., 2006; Gazi & Huppertz, 2015)

The present data also show that the solubility and browning index are not correlated at 25 °C (**SI- Table 2**) but they are inversely correlated at 40 °C (**SI- Table 3**). Previous studies have showed no correlation between the increase of the browning index and the loss of solubility of MCP (Nasser et al., 2018) whereas some other have suggested that MCP's solubility loss at 40 °C is related to the products formed during Maillard reaction (Le et al., 2011, 2013). At this stage, the relation between the solubility loss and the browning index rests unclear.

Quantification of proteins by High-Performance Liquid Chromatography (HPLC)

An alternative approach we used to assess the solubility loss during storage was the determination of the protein concentration in the supernatant of the solubilized MCP by HPLC. The caseins concentration (α-CN, β-CN, and κ-CN) and the total protein concentration (sum of α-CN, β-CN, and κ-CN) are depicted in **Figures SI- 3** and **SI-4**, respectively, where the lines are a guide for eye to help the reader and not a result of modelization of an explicit physical mechanism. In line with previous results, the protein concentration decreases (indicating difficulty of rehydration) more rapidly when powder storage is performed at 40 °C than at 25 °C for all MCP. The concentration of β-CN and κ-CN in the cross-linked MCP at t_s=0 days is considerably lower than that of the MCP-0 %, which is consistent with previous research that showed β-CN and κ-CN are more sensitive to cross-linking than α-CN (Hinz, Huppertz, Kulozik & Kelly, 2007).

The protein solubility (sum of α-CN, β-CN, and κ-CN) of MCP after a storage carried out at 25 and 40 °C displayed in **Figure SI-4a** reveals that the evolution of protein solubility of the MCP is dependent of the storage temperature. For a storage at both, 40 °C and 25 °C, the MCP solubility drastically decreases after 30 days of storage. After this storage time, the protein solubility of non-cross-linked and cross-linked MCP shows no significant changes over time.

Concerning how the cross-linking degree impact the protein solubility, **Figure SI-4b** shows that, regardless of the degree of cross-linking, all MCP stored at 40 °C exhibits a similar slope of protein solubility loss after 30 days of storage. For a storage at 25 °C (**Figure SI-4c**), the rate of protein solubility loss with aging is slower than it is occurring when storage was done at 40 °C, more specifically these differences can be noticed after 30 days of storage. At 30 days of storage at 25 °C, MCP-58% is more soluble than MCP-0, 36 and 48 %, but after a prolonged storage time, it shows a sharp decrease in protein solubility that is finally lower than for MCP-0 and MCP-36 % after 180 days of storage. This behavior suggest that an extensive cross-linking could decrease the loss of solubility during the first 30 days of storage at 25 °C due to a prevented physical interaction between the casein micelles but after a prolonged storage time, the physical interactions are beginning to affect the solubility.

One can also notice that MCP-48 and 58 % are slightly less soluble through storage than MCP-0 % and MCP-36 % at 40 °C even though they have they possess the higher lactose content (**Figure SI-4b and SI-4c**).

The solubility loss (Fig. 5) and the protein solubility loss (**Figures SI-3 and SI-4**) are positively correlated (**Table SI-2 and SI-3**). This suggests that the minerals and proteins dispersion from the MCP particles to the solvent occur simultaneously at least during the first rehydration h.

No correlation between the solubility loss and protein solubility loss and increase of the wetting time through storage was found (**Table SI-2 and SI-3**). The lack of correlation is probably because these two analyses measure different solubility characteristics during the rehydration process. As previously explained, wetting time would be related to the time needed for MCP particles to interact with the solvent and to be dissolved whereas the protein solubility loss measurement is related to the quantity of soluble protein after 1 h, even if not all micellar casein powders were released and dissolved.

Determination of covalent interactions of the insolubles during storage

The electropherograms of the pellet (insoluble material) after solubilization of the MCP after 0 and 180 days of storage are shown in Fig. 5. At t_s=0 days, one observes that MCP-0 % (lane 1) does not possess species with molecular weight >240 KDa. The formation of polymers with a molecular weight of more than 260 KDa that do not enter the gel is seen in the case of the cross-linked MCP (lanes 2–4). Besides, there is a progressive reduction of the bands between 25 and 40 KDa, attributed to the cross-linking of α-, β- and κ-casein.

After storage, t_s= 180 days at 40°C, one observes the formation of the species with a molecular weight >70 KDa for the MCP-0% (lane 5) that were not present before storage. For the cross-linked powders (lanes 6–8), little differences are noticed for high molecular weight species < 70 KDa, but the reduction of the width of the α-, β- and κ-casein bands indicate a cross-linking formation during storage.

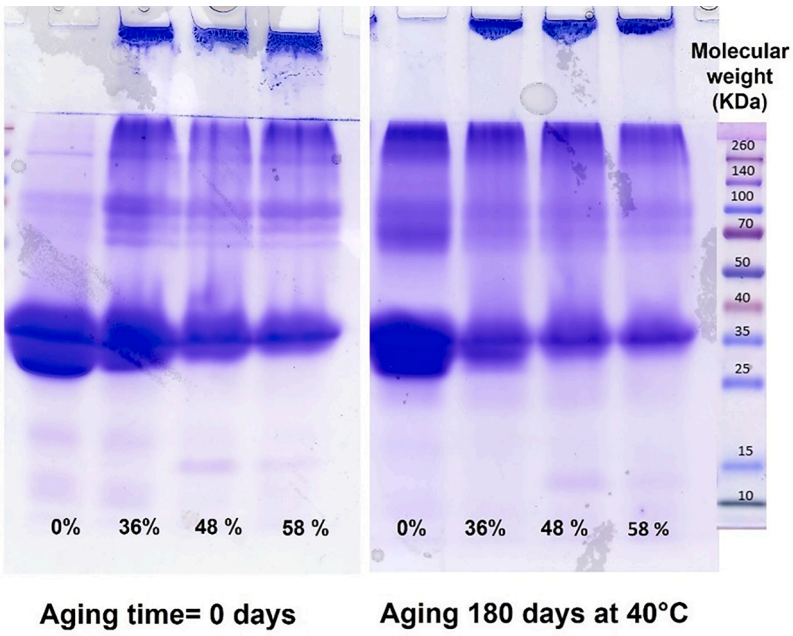

Fig. 5. Electropherograms under reduced conditions of non-cross-linked (0 %) and cross-linked MCP at different polymerization or cross-linking degree ΔDP (36, 48, 58 %) before (aging time = 0 days) and after storage (180 days at 40 °C). The lines used in are only a guide to the eye.

When the formation of species with a high molecular weight (Fig. 5) and the evolution of the wetting time through storage (Fig. 4) are compared, one can notice that even though MCP-0% clearly possess high molecular species after 180 days of storage, the impact of these species is almost non-existent in terms of wetting time. On the other hand, the wetting time of the cross-linked MCP varies greatly after storage, even though the cross-linking appearance (reduction of α-, β- and κ-casein bands) seems minimal. The reasons for the differences between wetting time and formation of covalent cross-linking evidenced by the SDS-PAGE results may be linked to the critical effect that larger polymers (that did not penetrate the gel) have in the increase of the wetting time. Anema (2006) suggested that MCP's solubility will not start to decline until a large amount of cross-linking has been formed. Also, it should be noted that SDS-PAGE was carried out in the pellet (insoluble material) whereas wetting time measured the occluded and interstitial air release from the MCP (soluble) particles. For that reason, it seems feasible to suggest that the decreased capacity of MCP to interact with the solvent is not only caused by the formation of covalent cross-linking but also due to non-covalent protein-protein interactions, such as the hydrophobic interactions between the casein micelle (Havea, 2006). Our hypothesis is that the initial polymerization degree would favor the development of casein protein-protein interactions between the casein micelles. From these results, we argue that the decrease of the solubility and the increase of wetting time are not directly caused by the covalent cross-linking of MCP during storage, but due to the indirectly increased physical interactions of cross-linked MCP when compared to non-cross-linked ones.

Summaries and conclusion

This study provided new information on the physical properties of cross-linked MCP during storage. The behavior of cross-linked and non-cross-linked MCP during rehydration was markedly affected by storage conditions. After only 30 days of storage at 40°C, the solubility of both the cross-linked and non-cross-linked MCP dramatically decreased. Changes in the browning index and the solubility indicated that the cross-linked MCP and non-cross-linked MCP age similarly. Wetting time determination showed that the initial cross-linking degree is the main cause of the reduced interaction between the powder particles and the solvent, which further decreases with storage, yet SDS-PAGE results indicated that other non-covalent protein-protein interactions may be involved in the increase of the wetting time during storage. The findings indicate that the initial level of cross-linking would cause modifications to MCP's functional characteristics, but the development of storage-induced cross-linking is unrelated to the evolution of MCP's functional characteristics.

CRediT authorship contribution statement

Angella Velazquez-Dominguez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Manon Hiolle:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology. **Marwan Abdallah:** Writing – review & editing, Formal analysis. **Amandine Descamps:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Guillaume Delaplace:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Paulo De Sa Peixoto:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Angella Velazquez Dominguez reports financial support was provided by French National Research Agency. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2024.100669.

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