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HAL Authorization

1 **Effect of the stabilizers on partial coalescence occurring in cocoa butter-in-water**
2 **emulsions: a comparative study between sunflower lecithin, buttermilk powder and**
3 **sodium caseinate.**

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10

11 Abstract:

12 The texture of an emulsion comprising crystallized dispersed phase depends on the extent of
13 partial coalescence. The aim of the present work was to determine the effect of three stabilizers
14 on partial coalescence and therefore on the texture of cocoa butter emulsions initially prepared
15 in the oil liquid state and allowed to crystallize for seven days. Partial coalescence was assessed
16 by visual inspection, granulometry and confocal laser microscopy. We show that the type of
17 emulsifier is of utmost importance, everything else being unchanged: dairy proteins are too
18 protective, inhibiting partial coalescence and the formation of a gel, lecithins are not protective
19 enough to lead to interesting gels while buttermilk powder, comprising a mixture of both
20 stabilizers, is very promising since a spreadable gel could be obtained thanks to connections

21 between droplets. Interfacial tension measurements, between water and middle chain
22 triglycerides that are liquid at room temperature, were carried out to highlight the comparative
23 behavior of the stabilizers. Atomic Force Microscopy (AFM) was used to map the interface
24 topography. In the case of buttermilk, the surface was rough with the existence of some
25 protruding crystals responsible for these connections. Wide Angle X-ray Scattering (WAXS)
26 and Small Angle X-ray Scattering (SAXS) were carried out to assess the lateral and longitudinal
27 packing of cocoa butter triglycerides in the emulsion droplets stabilized by the three emulsifiers.
28 All these results provide an understanding of the origin of the very different textures of the
29 emulsions obtained with the three stabilizers.

30

31 Keywords: O/W emulsions, partial coalescence, edge-piercing, solid oil, cocoa butter, butter
32 milk powder, sodium casinate, sunflower lecithin

33 1. Introduction

34 Emulsions are mixtures of two immiscible fluids, one being dispersed in the other one, which
35 is called continuous phase. When oil drops are dispersed in an aqueous phase, the emulsion is
36 said direct or oil-in-water (O/W). In the most general case, the two fluids are in a liquid state
37 so that, without any other constraint, drops are spherical to minimize their surface energy.
38 Emulsions are metastable systems destined to evolve with time through instabilities leading
39 either to solely macroscopic inhomogeneity without alteration of individual drops (flocculation,
40 creaming or sedimentation) or to a shift of the droplet size distribution (Ostwald
41 ripening(Ostwald, 1897) and coalescence (Leal-Calderon, Bibette, & Schmitt, 2007)) until
42 complete phase separation. Coalescence of liquid droplets corresponds to the fusion of droplets
43 and shape relaxation. Each coalescence event between two droplets ends with a single larger
44 and spherical droplet. The droplet size distribution evolves therefore by a shift towards a larger
45 average size and enlargement of the distribution. In many cases, in food or cosmetic products,
46 the dispersed phase of the emulsions is no more liquid but crystallizes. The crystallization of
47 fat influences the rheological and textural properties of finished products (van Vliet, van Aken,
48 de Jongh, & Hamer, 2009). When the dispersed oil becomes solid under cooling, a new
49 instability can occur called edge piercing or partial coalescence. Due to crystallization, the
50 droplet shape is no more governed by interfacial tension so that the droplets lose their pure
51 spherical shapes and adopt rough surfaces. In this case, coalescence is modified. The crystals
52 that form in the droplets can pierce the aqueous films between neighboring droplets and connect
53 them by forming lipid bridges, causing them to coalesce. However, the presence of enough

54 crystals within droplets also limits the relaxation shape of the connected droplets, as the crystals
55 stiffen the droplet structure (Boode & Walstra, 1993; Fredrick, Walstra, & Dewettinck, 2010).
56 The coalescence is therefore called partial. It may result in the formation of a percolated
57 network throughout the sample giving rise to an elastic dominant behavior (Thivilliers, Drelon,
58 Schmitt, & Leal-Calderon, 2006) or in other words to the emulsion gelling. Alternatively,
59 roughness of the particle surface may induce jamming due to a “gearing effect” without piercing
60 the interfacial film that is to say without forming true connections between the particles, just
61 correlations of positions and orientations. Jamming is also responsible for an elastic dominant
62 behavior of the sample (Giermanska et al., 2007; Holmes, Fuchs, & Cates, 2003; Thivilliers,
63 Laurichesse, Saadaoui, Leal-Calderon, & Schmitt, 2008). To differentiate between the two
64 gelling mechanisms of crystallizable oil emulsions may be challenging. In practice, to
65 demonstrate whether gelation is induced by either coalescence or jamming, it is usual to heat
66 the emulsion above the melting temperature of the fat in order to allow shape relaxation of
67 droplets. If melting the crystals leads to larger-diameter drops than in non-heated control or to
68 a macroscopic oil layer at the top of the sample, it indicates that connections (with a pore
69 between the drops allowing for a dispersed phase exchange) occurred, gelation was therefore
70 the result of partial coalescence. On the contrary, if the crystal fusion leads to sample
71 liquefaction and relaxation of droplet shape with no change in the droplet size distribution of
72 the emulsion, this means that gelation was the result of jamming alone. The mechanism can
73 therefore be assessed *a posteriori* by this simple heating. Partial coalescence has been largely
74 studied with milk fat, a very commonly used animal-based fat. Partial coalescence has been
75 shown to be influenced by many factors. Indeed, this mode of gelation is highly dependent on

76 the state of crystallization of the oil, *i.e.* the quantity of crystals (Thivilliers et al., 2008), their
77 shape, size and organization within the droplets (Wang, Hartel, Li, & Zhang, 2023). Stiff,
78 pointed crystals are able to pierce the interface and favor partial coalescence as opposed to
79 softer crystals. In this sense, polymorphism also has an influence on partial coalescence due to
80 the differences in rigidity and stability of the different crystalline forms. Crystals in the low-
81 density α form, whose organization is very close to the liquid form, are less apt to generate
82 coalescence compared with crystals in the β' form (Wang et al., 2023). There is an optimum
83 solid content to promote partial coalescence (Fredrick et al., 2010; Thivilliers et al., 2006;
84 Walstra, 1987). Thivilliers *et al.* have shown that the optimum solid content promoting the
85 partial coalescence, is around 15% corresponding to a temperature of 25°C, for anhydrous milk
86 fat or cocoa butter droplets stabilized by a mixture of sodium caseinate protein and Tween 20
87 surfactant. This optimum crystal content originates from a maximum probability of contact and
88 piercing between a solid and a liquid droplet surface. For lower crystal contents, liquid/liquid
89 contacts are favored while for higher crystal contents, solid/solid contacts are favored. None of
90 these types of contact leads to connections. The composition of the interface is also of utmost
91 importance, as crystals need to protrude enough and be able to pierce the film composed of
92 neighboring droplet surfaces. The sodium caseinate protein alone or adsorbed particles inhibit
93 the connections and are therefore too efficient protectors (Giermanska-Kahn, Laine, Arditty,
94 Schmitt, & Leal-Calderon, 2005; Thivilliers et al., 2008). In opposition, adding smaller
95 molecules as surfactant (Thivilliers et al., 2008), sucrose esters (Zeng et al., 2022) or mono-
96 and diglycerides (Wang, Hartel, & Zhang, 2021) may improve partial coalescence either by
97 their insertion between proteins or by their positive effect on the crystallization (Wang et al.,

98 2021). Numerous studies have addressed the relationship between partial coalescence and the
99 emulsions texture, especially with milk fat. Nevertheless, it remains challenging to finely tune
100 the phenomenon to impart texturing avoiding destabilization or macroscopic phase separation.

101 Cocoa butter, a main compound of chocolate, is made of triglycerides and exhibits 6
102 different polymorphs (Wille & Lutton, 1966) named from I to VI. The forms are also named
103 following their packing: α , β' and β correspond to a hexagonal, an orthorhombic and a triclinic
104 cell of the lateral chains packing respectively, while 2L or 3L corresponds to the longitudinal
105 arrangement of fatty acid chains in strata, the thickness of which reflects the number of
106 superimposed fatty acid chains (2 or 3). Wide Angle X-ray Scattering (WAXS corresponding
107 to the scattering q larger than 1 \AA^{-1}) allows assessing the cell structure that is to say the lateral
108 packing while Small Angle X-ray Scattering (SAXS corresponding to q vector varying between
109 0 to 1 \AA^{-1}) gives access to the longitudinal packing. Polymorph transitions from less stable to
110 more stable forms may occur under storage (van Malssen, van Langevelde, Peschar, & Schenk,
111 1999), (Afoakwa, Paterson, Fowler, & Vieira, 2009; Bloquet-Maurras, Bentaleb, Laurichesse,
112 Bayard, & Schmitt, 2023; Bricknell & Hartel, 1998). To allow comparison between samples,
113 we chose to set the aging time at 4°C at seven days.

114 The objective of the work, presented herein, was indeed to determine the effect of the
115 stabilizer on the partial coalescence in cocoa butter-in-water emulsions. The adopted strategy
116 was to formulate model systems varying only one parameter. Keeping both, the amount of fat
117 and the droplet size ($7\mu\text{m}$ in diameter) constant, three different stabilizers were considered:
118 sunflower lecithin (L), a plant-based stabilizer, sodium caseinate (P) an animal-based protein

119 from milk and a mixture of protein and phospholipids often used in food industry, namely
120 buttermilk powder (BP). The results are presented at different observation length scales: at the
121 macroscopic level with direct sample examination, at the microscopic level with confocal and
122 atomic force microscopies and at the nanoscale with X-ray scattering. Special attention is paid
123 on both the interface and the polymorphic form of the dispersed phase compared to its bulk
124 analog. A mechanism explaining the appearances of the different emulsions after crystallization
125 is proposed and discussed.

126

127 2. Materials and methods

128 2.1. Materials

129 Cocoa butter (CB) was provided from VALRHONA (Tain L'Hermitage, France), it is a mixture
130 of triglycerides. Supporting Information SI 1 shows the fatty acid and triglyceride composition
131 of cocoa butter, determined by gas chromatography at ITERG (Pessac, France). The amounts
132 of unsaturated and saturated fatty acids are 33% and 63% respectively. For interfacial tension
133 measurements, the solid fats were replaced by middle chain triglycerides (MCT, SEDNA,
134 France) which remained liquid at room temperature. The solid fat was replaced by a liquid oil
135 to facilitate the measurements and also because only a comparative study between the
136 stabilizers was of interest. MCT was chosen because it is composed of a mixture of triglycerides
137 as cocoa butter, but with smaller chain lengths (capric (C8) and caprylic (C10) chain lengths).
138 Sunflower oil, provided from Cargill (Hamburg, Germany) was used in a preliminary study to

139 determine the effect of stabilizer concentration and process parameters on the emulsion droplet
140 size and emulsion stability at 4°C when the oil remains liquid. It served as a guide for the
141 emulsion preparation. The sunflower lecithin (Cargill, France) is a standard de-oiled sunflower
142 lecithin. It is a low-molecular-weight surfactant ($< 800 \text{ g}\cdot\text{mol}^{-1}$). Its phospholipid profile was
143 determined by liquid chromatography by ITERG (Pessac, France) and is listed in Supporting
144 Information SI 2. It was composed of 57% of phospholipids. The sodium caseinate (Armor
145 Protéines, France) had a minimum protein content of 88% (supplier's data). Buttermilk powder
146 (Corman, Belgium) is an industrial product obtained by drying the aqueous phase of pasteurized
147 dairy cream, during the physical process of producing anhydrous milk fat. It contained 30.1%
148 protein, 50% lactose and 12.8% fat, including 6.49% phospholipids. The main proteins in
149 buttermilk powder are milk proteins, of which 80% are caseins and 20% are serum proteins.
150 The phospholipid profile of buttermilk powder is given in Supporting Information SI 3.

151 2.2. Methods

152 2.2.1. Emulsification

153 Emulsions were prepared at 60°C, well above the fusion temperature of cocoa butter, by a
154 progressive incorporation of the liquid fat in the aqueous phase using an Ultra-Turrax® T25
155 (IKA) mixer equipped with a S25 KV-25F shaft and operating at 12,000 rpm for 90 seconds.
156 The dispersed phase represented 35 wt% of the emulsion. Table 1 reports the detailed
157 composition of the emulsions. The milk protein or the buttermilk powder were added to the
158 aqueous phase (at a mass fraction with respect to the total emulsion mass of 3% for sodium
159 caseinate and 7.43% for buttermilk powder, respectively) while the sunflower lecithin (4 wt%

160 with respect to total emulsion) was added to the oil, prior to emulsion preparation. Once stirring
 161 stopped, the emulsions were transferred in tubes fully filled and placed in a thermostated
 162 chamber at 4°C, on a wheel to avoid creaming (protocol described in (Thivilliers et al., 2008)).
 163 The full completion of the vials allowed avoiding a large volume of air that would generate
 164 non-negligible stirring during storage. Emulsions were matured during seven days to allow
 165 crystallization and possible polymorphism transformations.

166 Table 1: Composition of cocoa butter-in-water emulsions stabilized by the three different
 167 stabilizers. The emulsion is composed of 65 wt% of continuous phase and 35 wt% of dispersed
 168 phase. The compositions are given in weight % with respect to the total sample.

169

Stabilizer	Composition of the aqueous phase		Composition of the lipophilic phase	
Sunflower lecithin	Pure water	65%	Cocoa Butter	31%
			Lecithin	4%
Sodium caseinate	Pure water	62%	Cocoa Butter	35%
	Sodium caseinate	3%		
Buttermilk powder	Pure water	53.57%	Cocoa Butter	35%
	Buttermilk powder	7.43%		

170

171 2.2.2. Droplet size distribution determination

172 The emulsion droplet size distributions were determined using a Mastersizer 3000 (Malvern).

173 The method is based on static light scattering and the Mie theory. The used refractive indexes

174 were equal to 1.333 for water and 1.469 for fat. Samples were diluted in an aqueous solution of

175 sodium dodecyl sulfate (SDS) at its critical micellar concentration of 8 mmol.L⁻¹. The dilution

176 was performed in presence of a low amount of surfactant to avoid any destabilization which

177 could introduce a bias in the measurement. SDS was chosen for its dissociating properties, as it

178 breaks the weak bonds between stabilizers that could cause attraction between emulsion
179 droplets. This allowed detecting only clusters obtained by partial coalescence rather than both
180 clusters due to partial coalescence and flocs. The diluted emulsion was introduced into the
181 dispersion cell filled with deionized water until laser obscuration reached 5%. This dilution
182 prevented multiple light scattering. Each measurement was performed three times. Droplet size
183 distribution was determined after preparation and after seven days storage at 4°C. The droplet
184 size distribution was the average of three consecutive measurements. The considered diameter
185 D was the volume-average diameter $D[4,3]$, where each drop was weighted by its volume:

$$186 \quad D = D[4,3] = \frac{\sum_i n_i d_i^4}{\sum_i n_i d_i^3} \quad (\text{Eq 1})$$

187 Where n_i is the number of droplets of diameter d_i . This diameter was chosen as it best
188 represented the way the fat was distributed in drops.

189 2.2.3. Optical microscopy

190 Optical microscopy allows a direct observation of the samples. It also allows to check the
191 consistency of the granulometry data that do not result from a direct observation but exhibit
192 excellent statistics. The samples were observed without dilution using an Olympus BX53
193 microscope equipped with x10 and x20 objectives. The images were collected thanks to a
194 camera (10.6 megapixels SC100, Olympus) and CellSens software. Image J software was
195 further used to set the scale bars.

196 2.2.4. Confocal microscopy

197 Samples were observed using a confocal laser scanning microscope (Zeiss LSM 800, Carl Zeiss
198 SAS, France), without dilution at 10°C using a Peltier temperature-controlled stage. Nile Red
199 ($\lambda_{\text{excitation}} \sim 590 \text{ nm}$, $\lambda_{\text{emission}} \sim 650 \text{ nm}$) was used to stain the fat. Emulsions were formulated
200 directly with the initially colored fat phase. Emulsions were then observed after 7 days of
201 crystallization, with the x10 objective (dry objective) and with the x63 objective (oil immersion
202 objective). Images were collected and saved thanks to Zeiss ZEN. Image J software was further
203 used to set the scale bars.

204 2.2.5. AFM

205 The topography of the interface was studied for different emulsifiers by atomic force
206 microscopy following the protocol described by Thivilliers (Thivilliers et al., 2008). For this
207 purpose, a drop (approximate volume 4 μL) of cocoa butter, previously melted at 60°C was
208 deposited on a graphite substrate freshly cleaved. This oil laden substrate was then inverted and
209 brought into contact with the aqueous phase containing sodium caseinate or buttermilk powder.
210 In the case of sunflower lecithin-based interfaces, a drop of cocoa butter containing sunflower
211 lecithin was brought into contact with pure water. The samples, cooled to 4°C and stored at this
212 temperature for seven days, were then observed using an atomic force microscope (Dimension
213 Icon®, Bruker), at 4°C, thanks to a Peltier temperature-controlled stage under nitrogen
214 environment to prevent condensation. Samples were imaged using intermittent contact mode
215 (tapping) with a silicon Tespa-300 probe (Bruker). Dark colors represent the lower parts of the
216 sample, while light colors represent the higher parts of the sample. Images were acquired line
217 by line at a frequency in the Hertz range.

218 2.2.6 Interfacial tension measurements

219 As fats are solid at room temperature, middle chain triglyceride (MCT, density at 20°C =
220 0.9458 g.cm⁻³) oil was chosen in order to perform the measurements without heating. Even if
221 the oil was not exactly the same as in the emulsions, this allowed a comparison between the
222 stabilizers independently of the crystallization process. The device was a Tracker tensiometer
223 (Teclis). A drop of aqueous phase or pure water (in the case of lecithin) was formed at an inox
224 G18 tip of a syringe and plunged in a quartz cuvette filled with pure oil or oil containing lecithin.
225 The aqueous drop volume (5 µL) was maintained constant for all measurements. The drop shape
226 was then analyzed using the Windrop software and the interfacial tension between the two
227 liquid phases was then extracted. All measurements were performed at 20°C using a
228 thermostatically-controlled bath.

229 2.2.7. X-ray scattering

230 A Xeuss 2.0 diffractometer (Xenocs, France) equipped with a GeniX-3D copper microsource
231 ($\lambda_i = 1.54 \text{ \AA}$) and a Dectris Pilatus 300 k detector operating in vacuum was used for X-Ray
232 scattering experiments. The detector was positioned at a distance of 174.5 mm, giving access
233 to a 2θ range between 1.2° and 29°. The oils or the emulsions were introduced in 1 mm diameter
234 capillaries at 60°C. After sealing, the capillaries were left to crystallize at 4°C during seven
235 days before measurements. The “Foxtrot” software was used to transform the data from
236 reciprocal into direct space. Raw data images were processed with Java-based graphics software
237 developed by Xenocs and the Synchrotron SOLEIL. The instrument software constructed a

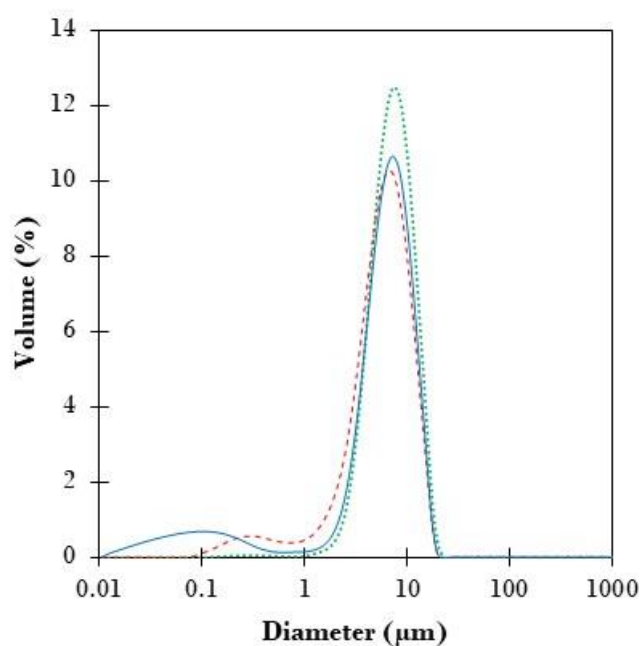
238 histogram of signal intensity as a function of 2θ . Polymorphs were determined according to
239 their short d-spacing characteristics (\AA).

240

241 3. Results

242 3.1. Cocoa butter-in-water emulsions after preparation (at 60°C)

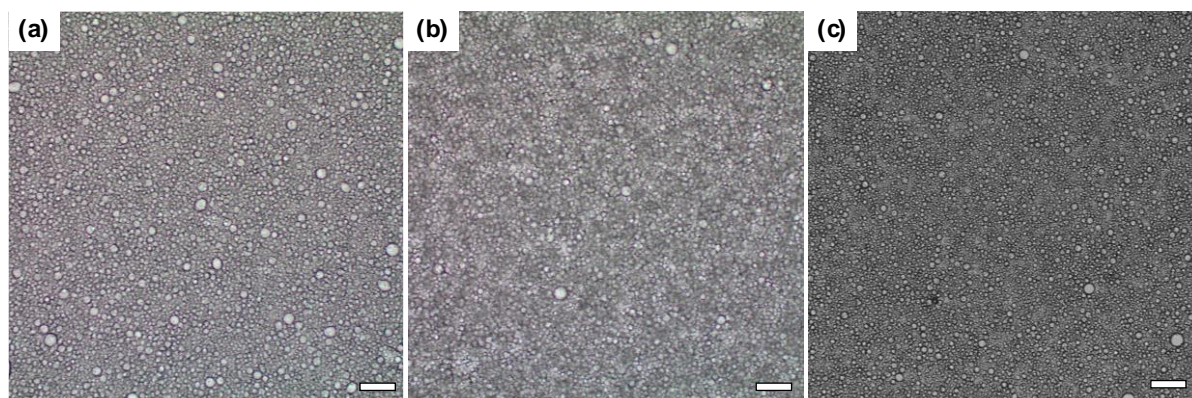
243 A preliminary study allowed determining the composition parameters in order to produce
244 emulsions with comparable average sizes of the order of $7\ \mu\text{m}$. The compositions are noted in
245 Table 1 and the droplet size distributions are reported in Figure 1 as well as optical microscopy
246 pictures of the emulsions.



247

248

249



250 Figure 1: Droplet size distributions of the emulsions (top) and corresponding microscopy
251 pictures (bottom) just after preparation at 60°C, before crystallization. The stabilizer is
252 sunflower lecithin (red dashed line and a), sodium caseinate (dotted green line and b) and
253 buttermilk powder (blue line and c). The exact compositions are given in Table 1. The scale bar
254 represents 20 µm.

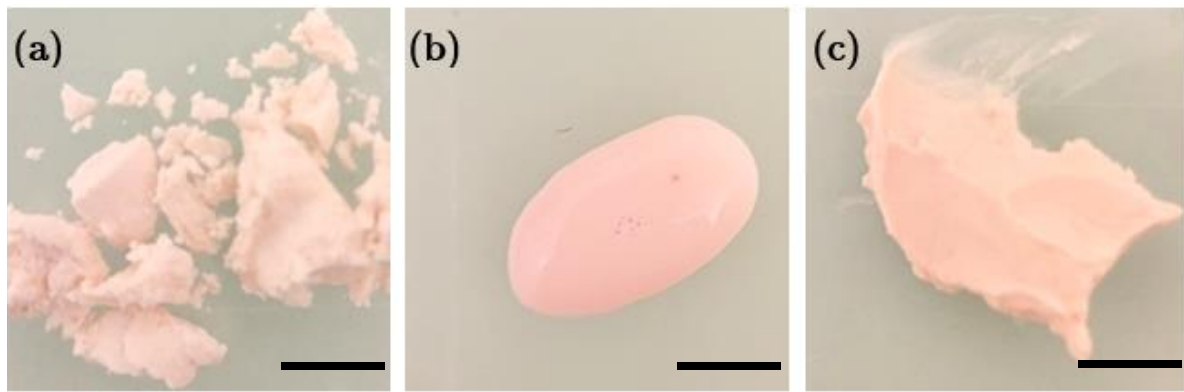
255

256 After preparation, the emulsions were stored at 4°C for seven days as described in the methods
257 section.

258 3.2 Cocoa butter-in-water emulsions after 7 days of crystallization at 4°C: macroscopic scale

259 3.2.1. Appearance at 4°C

260 After storage at 4°C, an aliquot of the emulsions was withdrawn with a spatula and a simple
261 spreadability test was performed. While the emulsions stabilized by the three different
262 stabilizers were very similar in terms of droplet size distribution and fluidity, immediately after
263 preparation at 60°C, clear differences appeared after the storage period at 4°C, showing that the
264 emulsions had evolved differently (Figure 2). It should be noted that the droplet size distribution
265 of equivalent sunflower oil-in-water emulsions (serving as controls) did not evolve significantly
266 at 4°C over a period of 7 days (Supporting Information SI 4) showing the importance of the oil
267 solid state on the resulting emulsion appearance.



268

269 Figure 2: Photographs of 35% cocoa butter-in-water emulsion gels obtained after 7 days
270 crystallization at 4°C, stabilized with (a) sunflower lecithin, (b) sodium caseinate and (c)
271 buttermilk powder. The scale bar represents 0.5 cm. The pink color is due to the addition of
272 Nile Red in the cocoa butter prior to emulsification.

273

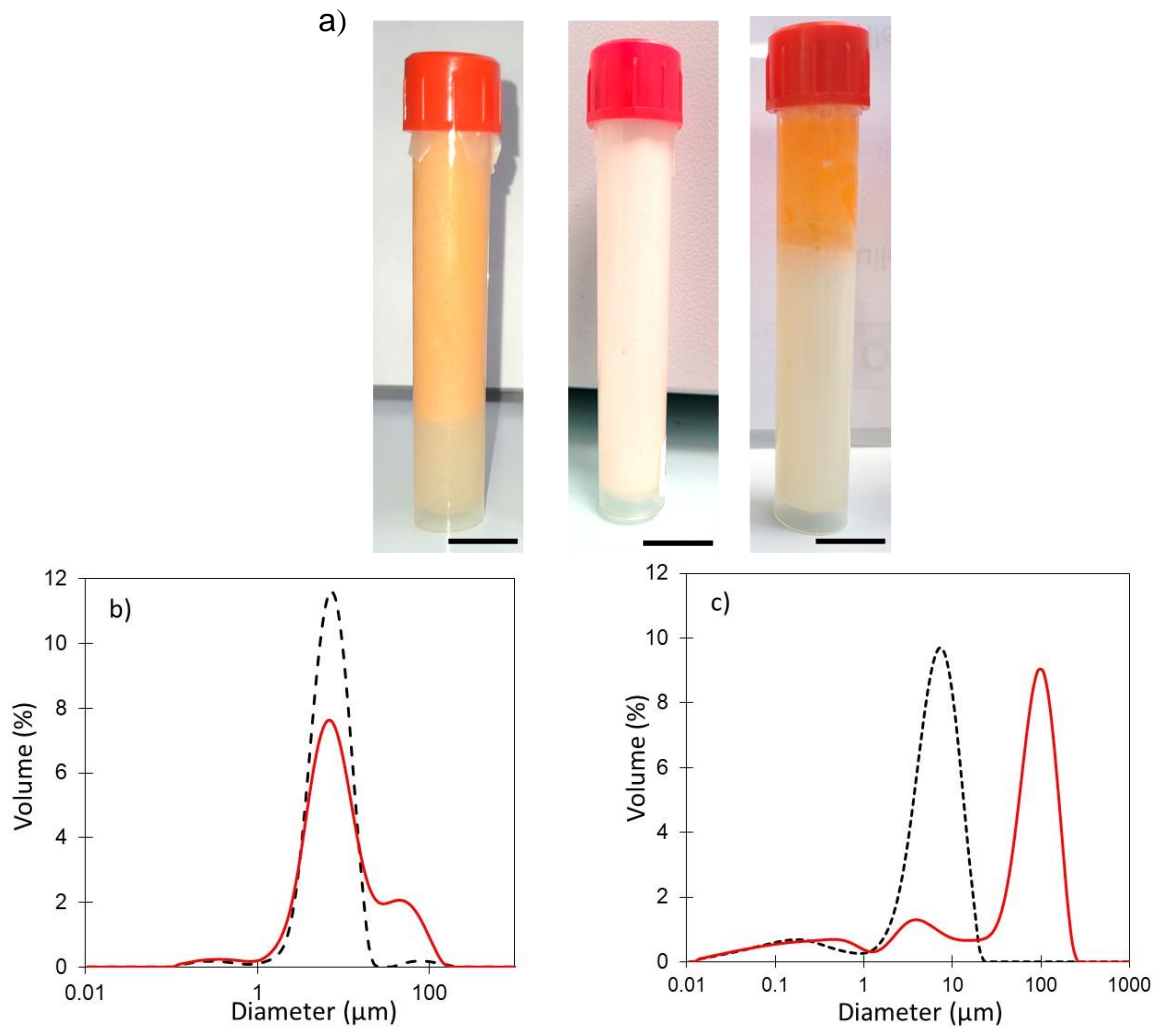
274 The emulsion stabilized by sunflower lecithin exhibited a very hard, brittle and non-spreadable
275 texture showing a very marked change in the emulsion during storage at 4°C (Fig. 2a). The
276 emulsion initially stabilized by sodium caseinate kept a liquid appearance after storage for 7
277 days at 4°C showing that the emulsion had not gelled (Fig. 2b). The emulsion initially stabilized
278 by buttermilk powder changed appearance after storage for 7 days at 4°C showing that the
279 emulsion had gelled since the sample did not flow anymore under its own weight, exhibiting an
280 interesting spreadability (Fig. 2c).

281

3.2.2. Appearance after heating at 50°C

282 In order to assess the possible gelling mechanisms that are responsible for the change of the
283 appearance of emulsions, the emulsions were heated at 50°C, above the melting temperature of
284 cocoa butter as usually done in literature (see introduction). Their respective aspects are shown

285 in Figure 3. When a white emulsion was present, the droplet size distribution was determined
286 and compared to the initial ones. They are reported in Figure 3b and c.



287 Figure 3: a) Macroscopic appearance of the 7 days crystallized emulsions (without Nile Red)
288 after heating at 50°C. Emulsion-stabilized by sunflower lecithin (on the left), sodium caseinate
289 (in the middle) and buttermilk powder (on the right). The scale bar represents 1 cm, b) droplet
290 size distribution of the initial (dotted line) and crystallized and heated emulsion stabilized by
291 sodium caseinate (red continuous line) and c) droplet size distribution of the initial (dotted line)
292 and crystallized and heated emulsion stabilized by buttermilk powder (red continuous line).

293

294 For the emulsion stabilized by sodium caseinate, there was no macroscopic oil layer released at
295 the top of the sample that remained white (Fig. 3a middle) and the size distribution hardly varied
296 (Fig.3b). This means that the oil was still dispersed in the form of an emulsion and that droplets
297 kept their individuality and did not connect, during the whole cycle, neither during
298 crystallization nor during heating.

299 In the emulsion stabilized by buttermilk powder, after 7 days of crystallization, a macroscopic
300 phase separation was visible after heating, (Fig. 3a right). This separation occurred between an
301 upper yellow phase (same color as heated cocoa butter) only composed of melted cocoa butter
302 and a white sub-phase composed of a dilute emulsion whose size distribution had evolved (Fig.
303 3c). This could indicate that the gelling mechanism responsible for the texture evolution is edge
304 piercing involving connections between drops. The proportion of the clear oil layer is about
305 33% so that edge piercing is substantial in this emulsion. Moreover, the droplet size distribution
306 of the remaining emulsion has changed compared to the initial one.

307 When the hard and brittle crystallized emulsion stabilized by lecithin was heated at 50°C, the
308 sample phase-separated (Fig. 3a left) into an upper yellowish phase, which did not correspond
309 to the color of heated pure cocoa butter and a sub-phase. The sub-phase was not limpid and was
310 colored. It was between whitish and yellowish with a granular aspect. The proportion of the
311 upper phase is of the order of 76 vol%, a value much higher than the proportion of cocoa butter
312 in the sample. This means that the upper phase also contained a large proportion of the total
313 water. The color of the upper phase was far from white (in contrast to the cocoa-butter-in-oil
314 emulsions). It exhibited a color close to the one of cocoa butter suggesting a cocoa butter

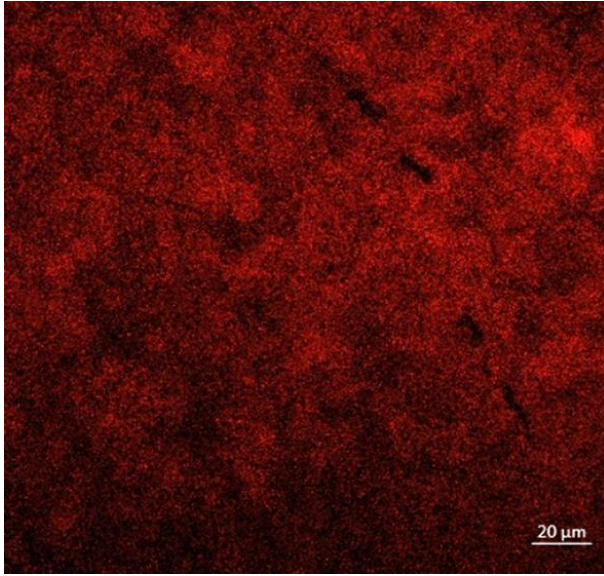
315 continuous phase. But as the color was attenuated, it indicates that this phase scattered light,
316 meaning that the phase is optically non homogenous or in other words it is a dispersion. It was
317 nevertheless not possible to distinguish whether the continuous phase of the whole sample was
318 aqueous or oily with the classical solvent test (test of dispersing a sample of the upper phase
319 with water or with oil). The structure of the cocoa butter-in-water emulsion stabilized by
320 sunflower lecithin that had been stored at 4°C for 7 days remained therefore somewhat
321 ambiguous at this stage.

322 3.3 Cocoa butter-in-water emulsions after 7 days of crystallization at 4°C: microscopic and
323 nanoscopic scales

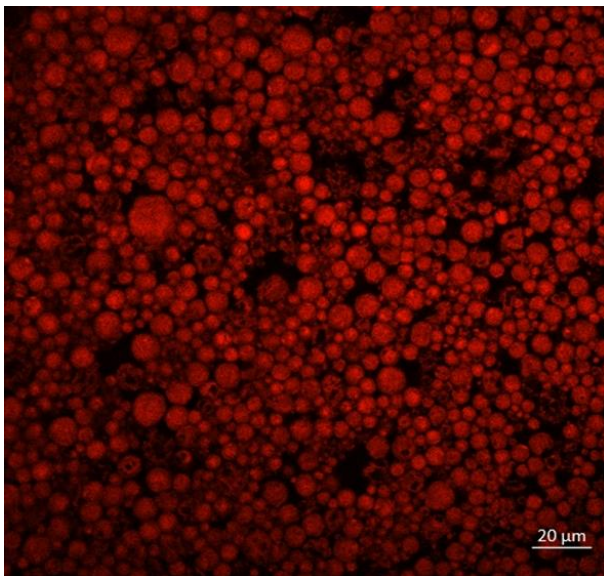
324 3.3.1. Visualization in the direct space by confocal microscopy
325

326 In order to get a better insight into the sample avoiding any bias originating from heating, the
327 crystallized fluorescent samples, containing Nile Red to stain the oil phase, were observed
328 under confocal microscopy. The pictures are reported in Figure 4.

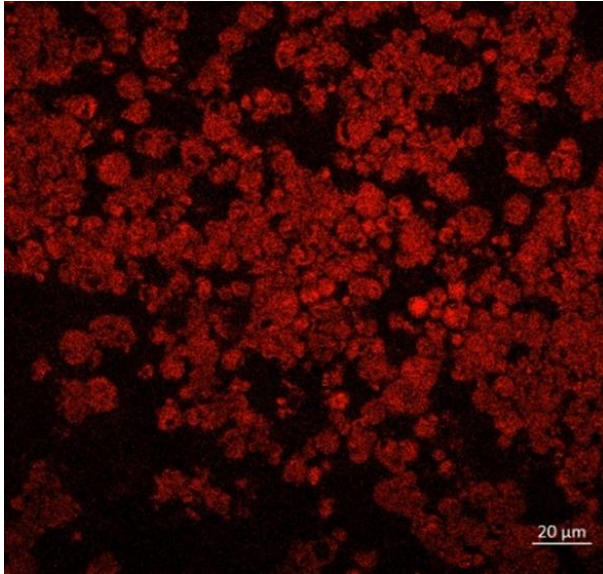
a)



b)



c)



329 Figure 4: Confocal microscopy images of cocoa butter-in-water emulsions, stabilized with a)
330 sunflower lecithin, b) sodium caseinate and c) buttermilk powder, after 7 days of storage at 4°C.
331 The initial droplet size was 7 μm for all the emulsions. The scale bar is 20 μm.

332

333 Again, the appearance of the three samples was very different.

334 In the presence of sodium caseinate, the droplets were clearly visible and generally individual
335 (Fig. 4b). The crystallized fat droplets did not connect and did not form a colloidal network. No
336 partial coalescence was observable.

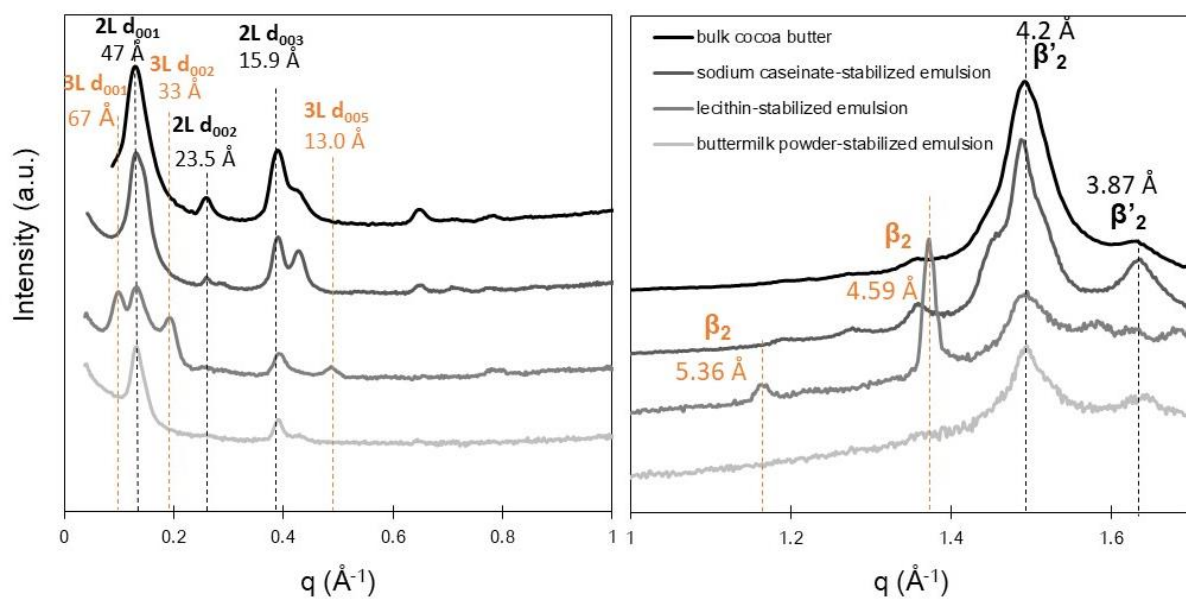
337 In the presence of buttermilk powder, the droplet network was clearly visible. The drops were
338 globally connected to each other, and a few individual droplets were noticeable (Fig. 4c).
339 Connections had therefore percolated throughout the sample.

340 In the presence of sunflower lecithin, confocal microscopy images showed large domains of
341 fatty material (red), with no visible drop boundaries (Fig. 4a). The 7 μm droplets seemed to
342 have completely disappeared. The fat appeared to be continuous. Uncolored domains

343 corresponding to water were also observed. A continuous network of fatty phase containing
344 water was therefore observed.

345 3.3.2 Characterization in the reciprocal space (SAXS and WAXS)

346 X-ray scattering was performed to characterize the crystallized fat in bulk and in the emulsions
347 (Figure 5).



348 Figure 5: SAXS (left) and WAXS (right) spectra of, from darker to lighter grey lines, bulk
349 cocoa butter, emulsions stabilized by sodium caseinate, emulsions stabilized by sunflower
350 lecithin and emulsions stabilized by buttermilk powder after 7 days of crystallization at 4°C.
351

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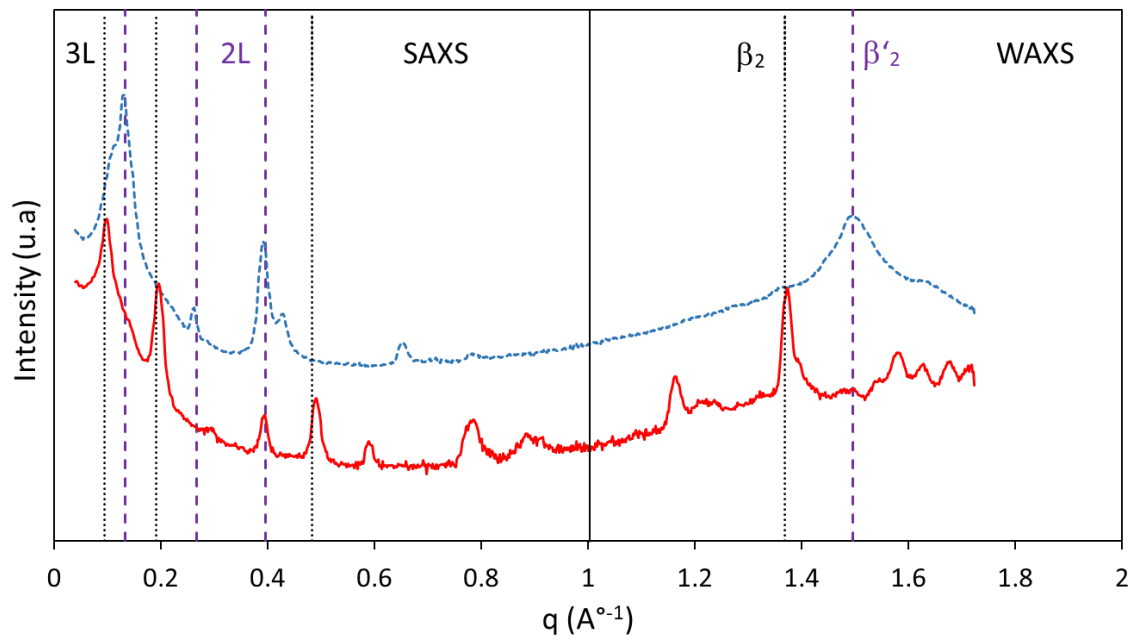
353 Figure 5 shows that the crystallization of the emulsion stabilized by sodium caseinate or
354 buttermilk powder led to the same polymorph as bulk cocoa butter. Three peaks were visible
355 (WAXS) at distances of 4.2 \AA , 4.6 \AA and 3.87 \AA , characteristic of the orthorhombic β'_2 structure

356 (Hicklin et al., 1985). SAXS analysis showed the 2L packing with a first peak corresponding
357 to a 47 Å distance. Thus, in bulk, or in both emulsions cocoa butter had an orthorhombic β'_2
358 crystal (WAXS) and 2L packing (SAXS) corresponding to the polymorph III.

359 In the case of the pure lecithin-stabilized emulsion, the spectra were more complex with the
360 coexistence of two polymorphs. Cocoa butter crystal in this emulsion exhibited two diffraction
361 peaks (WAXS) characterizing the orthorhombic β'_2 structure at 4.2 Å and 3.87 Å (the same as
362 in the bulk cocoa butter) as well as two peaks characterizing the triclinic β_2 structure at 5.36 Å
363 and 4.59 Å (Hicklin et al., 1985). The SAXS signal exhibited a peak at 67 Å corresponding to
364 an elementary distance of d_{001} , attributed to the longitudinal 3L packing of the cocoa butter
365 triglycerides. The peak at 47 Å (same as cocoa butter in bulk) corresponds to an elementary
366 distance of d_{001} , attributed to the longitudinal 2L packing. As a conclusion, the cocoa butter
367 dispersed in emulsions stabilized by sunflower lecithin crystallized under two coexisting crystal
368 polymorphs: β'_2 -2L and β_2 -3L corresponding to a coexistence of polymorphs III and V.

369 The nanostructure of cocoa butter crystals, matured for seven days at 4°C, was therefore not
370 modified by sodium caseinate, nor by buttermilk powder, but was influenced by lecithin. In this
371 case, sunflower lecithin would act as a nucleation support to form more stable β -nuclei. This
372 could be due to the fact that lecithin was added in the fat phase. It has been previously observed
373 that minor products can modify the crystallization of anhydrous milk fat (Bayard, Cansell, &
374 Leal-Calderon, 2022; Bayard, Leal-Calderon, & Cansell, 2017). To check our hypothesis on
375 the role of sunflower lecithin, a mixture of 4 wt% of sunflower lecithin in bulk cocoa butter

376 was prepared at 60°C and left to crystallize at 4°C for 7 days. The X-ray data are reported in
377 Figure 6.



378
379 Figure 6: X-Ray scattering spectra of cocoa butter containing 4 wt% of sunflower lecithin after
380 crystallization during 7 days at 4°C (blue dashed line, upper curve) and 7 more days at 25°C
381 (red line). As guides for the eyes, dotted black and purple dashed lines mark the position of the
382 3L and 2L packing for SAXS and the β_2 and β'_2 crystal forms for WAXS, respectively.

383

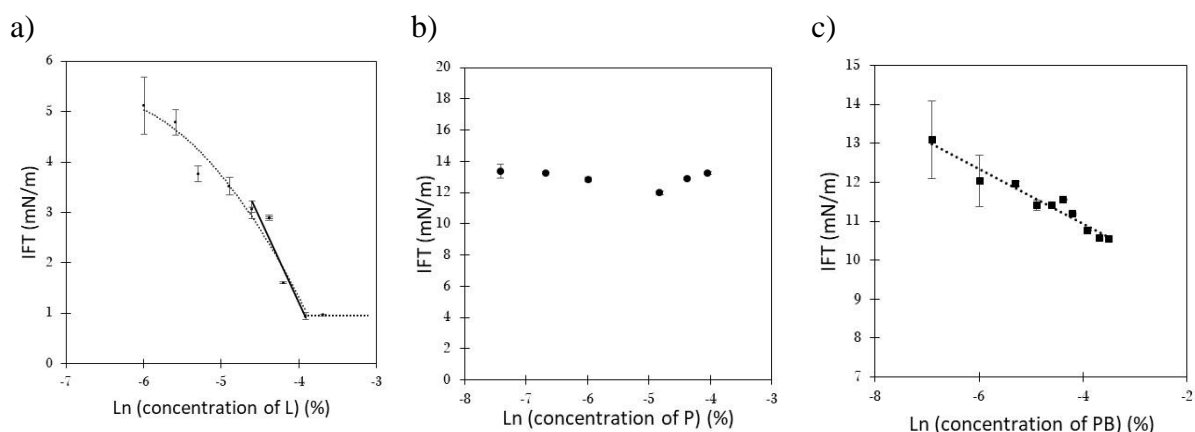
384 The SAXS experiments performed on bulk mixture of lecithin and cocoa butter in the same
385 proportion as in the emulsion and left to crystallize at 4°C for 7 days show the dominant
386 presence of 2L packing. Nevertheless, crystals with a 3L packing were also present in a much
387 lesser extent than in the corresponding emulsion. The WAXS experiments exhibited a
388 predominant peak corresponding to the orthorhombic β'_2 crystals with a secondary much less
389 intense peak corresponding to the triclinic β_2 crystals. To sum up, in the bulk lecithin-cocoa
390 butter mixture, there was a coexistence between the β'_2 -2L (same as in pure cocoa butter) and

391 β_2 -3L polymorphs as it was the case in the emulsion. However, contrary to the emulsion, the
392 β_2 -3L was hardly visible so that it may be less present in the bulk than in the emulsion. The
393 same mixture has then been heated to 25°C and left to age for 7 additional days. Again, X-Ray
394 diffraction indicated the coexistence of these two types of crystals with a higher occurrence of
395 the β_2 -3L polymorph. These scattering experiments, therefore show that lecithin modifies
396 crystallization in the bulk of cocoa butter by adding β_2 -3L crystals to the β'_2 -2L crystals that
397 are the only ones present in pure cocoa butter. In the lecithin emulsion, the same two
398 polymorphs coexist in the same way as in the bulk phase, whereas the β_2 -3L crystals are more
399 visible in the emulsion than in the bulk.

400 3.4 Characterization of the interface

401 3.4.1. Stabilizer adsorption

402 The first step was to compare the ability of the three stabilizers to adsorb at the interface
403 between water and a liquid triglyceride oil. In order to facilitate the experiments and avoid
404 heating, we chose to use middle chain triglyceride (MCT) that remained liquid at 20°C.
405 Eventhough the oil was not the one used for the emulsion study, it allowed a comparative study
406 of the water-oil interfacial tensions between the three emulsifiers. Figure 7 shows the evolution
407 of the interfacial tension IFT as a function of stabilizer concentration for the three stabilizers
408 taken at 1000 seconds in the steady state.



409 Figure 7: Interfacial tension between MCT oil and water at 20°C versus Neperian logarithm of
 410 the concentration of stabilizer expressed in weight %, a) for sunflower lecithin, b) for sodium
 411 caseinate and c) for buttermilk powder. Dotted lines are guides for the eyes. The straight line in
 412 (a) is the tangent at CMC allowing extracting the excess surface Γ (see text).

413

414 The three stabilizers behaved differently. Sunflower lecithin exhibited the classical evolution
 415 of surfactant stabilizer (Fig.7a). From this evolution, a critical micellar concentration (CMC,
 416 corresponding to the appearance of the plateau) could be extracted. It is equal to 2 wt% with
 417 respect to the MCT oil. The slope (-3.35 mJ/m^2) just before CMC gives access to the excess of
 418 surface (Gibbs, 1931) $\Gamma = 1.36 \cdot 10^{-6} \text{ g/m}^2$ with the hypothesis that the lecithin is globally neutral.
 419 Considering the molar mass of sunflower lecithin, 779 g/mol (Morrison, 1981), it can also be
 420 converted into a polar head per molecule equal to $12.2 \pm 2.3 \text{ nm}^2$, which is the surface occupied
 421 by one lecithin molecule at the water/oil interface. This value is close to the one (10.60 nm^2)
 422 determined for soy lecithin dissolved in water against paprika oleo-resin (Flores-Andrade et al.,
 423 2021).

424 The interfacial tension did not vary in the explored range (0.06 to 1.75 wt% with respect to the
 425 aqueous phase) in presence of sodium caseinate with an interfacial tension of the order of

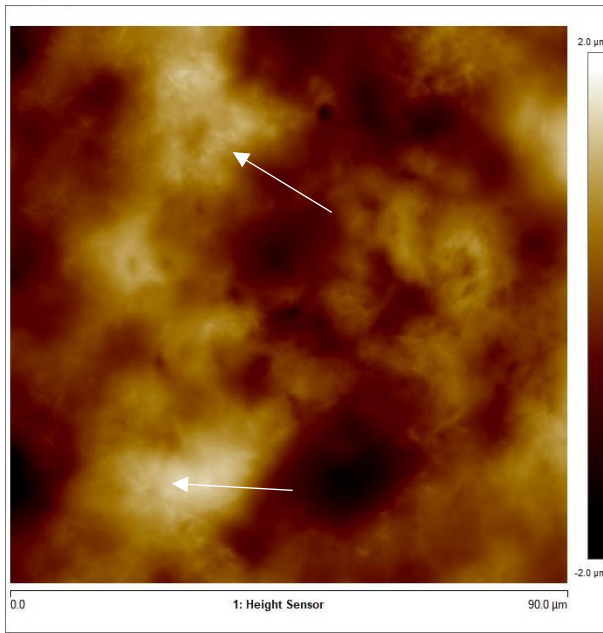
426 12 mN/m, meaning a saturated interface (Fig.7b). This tension is much higher than the one with
427 lecithin above the CMC, likely due to the much larger size of the protein compared to the
428 phospholipid and therefore to the lower possible surface density for proteins.

429 The evolution of the water/MCT interfacial tension in presence of buttermilk powder is shown
430 in Figure 7c. It varied between 13 and 8 mN/m depending on the concentration of buttermilk
431 powder in the aqueous phase (0.1 to 8 wt% with respect to the aqueous phase). These values
432 are slightly below that of caseinate, but much higher than that of lecithin. As buttermilk powder
433 is a mixture of proteins and phospholipids, the interface is not purely composed of one or the
434 other, but is a mixed interface. Due to the complex mixture of this real product, the
435 concentration of each component present at the interface could not be deduced. However, there
436 is co-adsorption of proteins and phospholipids, with probably a lower total surface density of
437 adsorbed objects than that obtained with sunflower lecithin and roughly similar to that obtained
438 with sodium caseinate.

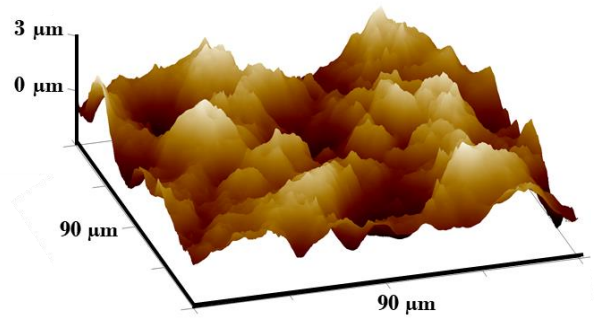
439 3.4.2. Interface topography by AFM

440 To understand the structure of oil/water interfaces in cocoa butter emulsions, measurements of
441 interface topography were carried out using atomic force microscopy on a macroscopic sample
442 prepared at 60°C and left to crystallize at 4°C. As for previous experiments, sunflower lecithin
443 was dissolved in the butter while the protein and the buttermilk powder were previously
444 dissolved in water (see methods for a more detailed description of the sample preparation). The
445 topographic images as well as 3D representations are reported in Figure 8. Some zooms are
446 available in Supporting Information SI 5.

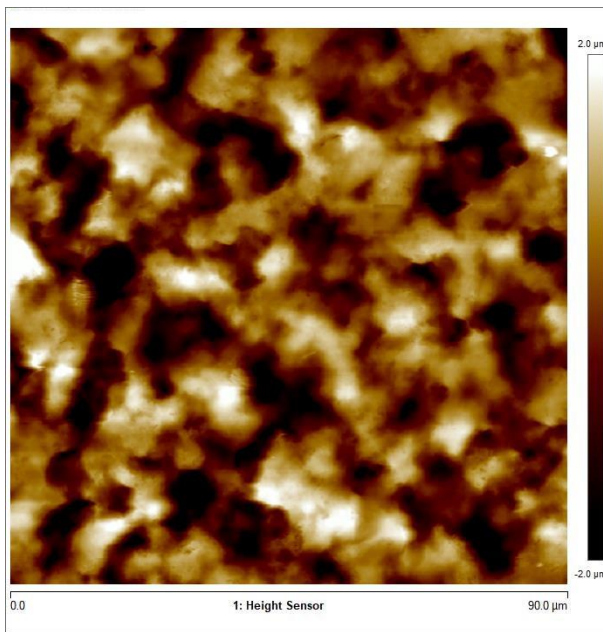
a)



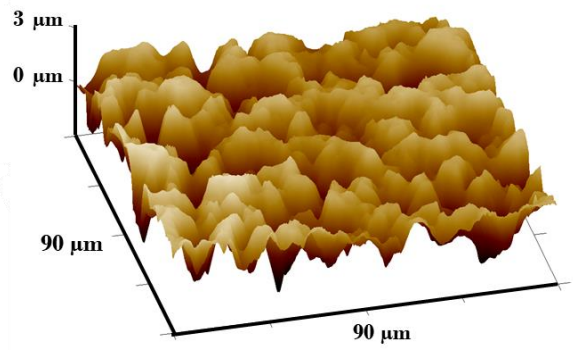
d)



b)

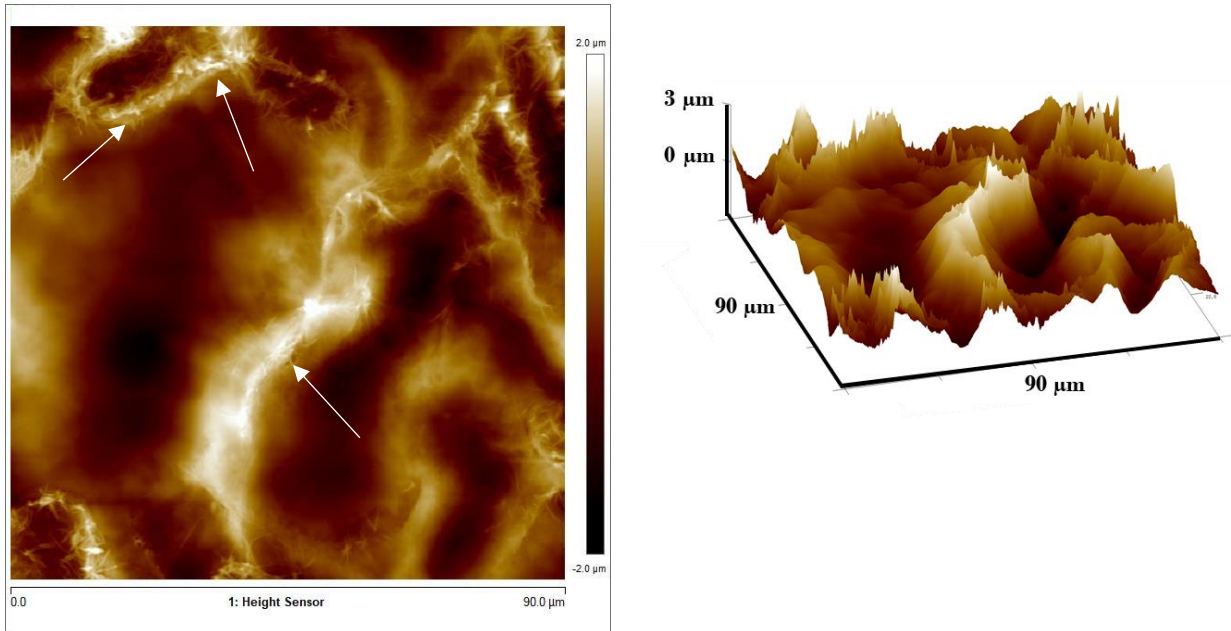


e)

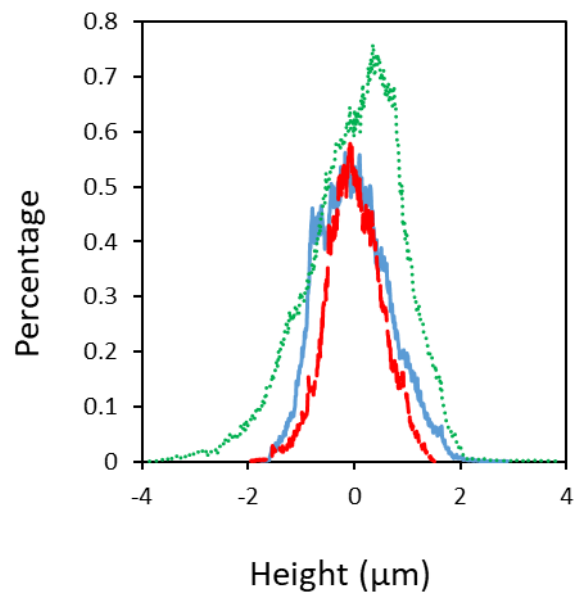


c)

f)



g)



447 Figure 8: Atomic force microscopy 2D (a-c) and 3D (d-f) pictures at 4°C of the surface of a
 448 macroscopic drop of cocoa butter in contact with water in the presence of (a, d) 4% sunflower
 449 lecithin, (b, e) 3% sodium caseinate solubilized in water (c, f) 7.43% buttermilk powder
 450 solubilized in water. In all cases, the topography is probed from the water side. The height scale
 451 is given on the right of the 2D pictures and is the same in all three cases. White arrows point

452 some crystals. (g) Height distribution of the whole micrographs. The stabilizer is sunflower
453 lecithin (red dashed line), sodium caseinate (dotted green line) and buttermilk powder (blue
454 line).

455

456 Even if the images were taken at the surface of a larger drop than the ones composing the
457 emulsions, comparison is informative. The images revealed differences between the three
458 stabilizers. In the presence of sodium caseinate (Fig. 8b and 8e), the surface showed significant
459 height variations. The surface roughness was the highest and the distribution was the largest
460 among the three systems. The surface roughness of the interface is the result of the presence of
461 crystals but no crystal could be distinguished at the surface even when zooming in (Supporting
462 Information SI 5b). In the presence of sunflower lecithin, height variations were also evidenced
463 by AFM. Moreover, some crystal can be distinguished (Fig. 8a and 8d) as highlighted by white
464 arrows and even better visible in Supporting Information SI 5a. In the presence of buttermilk
465 powder, height variation was also observed, of the same range than with lecithin and numerous
466 crystals can be seen throughout the picture (Fig. 8c and 8f and Supporting Information SI 5c).
467 Table 2 gives the maximal amplitude z_{\max} (difference between the maximal height and the
468 maximal depth) and the quadratic mean roughness R_q over the whole scanned surface, extracted
469 from micrographs reported in Fig. 8.

470

471 Table 2: quantitative characterization of the surface roughness deduced from AFM: maximal
472 amplitude difference z_{\max} and surface roughness R_q .

Stabilizer	Lecithin	Sodium caseinate	Buttermilk powder
z_{\max} (μm)	3.4	6.9	4.6
R_q (nm)	521	912	658

473

474

475 4. Discussion

476 All these results provide a clear picture of the mechanism operating for 7 μm -sized
 477 emulsions after storing seven days at 4°C, for each stabilizer.

478 4.1 Sodium caseinate

479 After crystallization, the emulsion remained liquid, and after one complete freeze-thaw cycle,
 480 no phase separation could be observed. The droplet size distribution was almost unchanged
 481 showing the high stability of the emulsions despite the transitions (crystallization and melting)
 482 occurring in the dispersed phase. The crystal polymorph (β'_2 -2L) in the droplets was the same
 483 as in the bulk phase as revealed by X-Ray scattering. Moreover, despite the highest interface
 484 roughness, no crystal could be observed as if the protein layer would cover and adapt to the
 485 surface roughness. The absence of crystal at the top of the surface is likely responsible of the
 486 absence of partial coalescence as schematically drawn in Figure 9a so that the liquid behavior
 487 is preserved after storage at 4°C. The stability against edge piercing is also confirmed by the
 488 absence of connections in confocal microscopy observations. The cocoa butter emulsion solely
 489 stabilized by proteins is stable against a complete freeze-thaw cycle. Crystals are unable to

490 break through the interfacial cohesive film, due to the proteins that most likely form thick,
491 viscoelastic layers on the surface of the droplets (Goff, 1997). This result is very similar to the
492 one obtained with anhydrous milk fat (Thivilliers et al., 2008) and proteins alone.

493 4.2 Sunflower lecithin

494 Things are very different for the emulsion stabilized by sunflower lecithin, as the sample after
495 crystallization at 4°C was solid, very hard and brittle (Fig. 2a). The sample was difficult to
496 disperse when cold. Confocal microscopy seems to indicate that the fat, stained by Nile Red,
497 constituted a continuous phase after crystallization. These observations lead us to hypothesize
498 that cocoa butter crystals destabilized the emulsion and resulted in phase inversion. Phase
499 inversion may result from the occurrence of very numerous connections between the former
500 droplets (scheme Fig. 9b). The continuous phase made of crystallized cocoa butter would
501 explain the very solid texture of the system. Water domains appearing black in confocal
502 microscopy observations, are trapped in the fat network. Knowing the structure at 4°C, one
503 better understands the observation after adding a heating step (Fig. 3). The lower phase is likely
504 composed of some dilute water-continuous emulsion remaining after a complete freeze-thaw
505 cycle, and the upper phase is likely composed of a yellowish oil continuous phase comprising
506 water domains that scatter light. Considering the proportions of the two phases, almost all the
507 cocoa butter is in the upper phase as well as a large proportion of the water.

508 SAXS and WAXS experiments show that the cocoa butter crystal polymorph in the droplets is
509 not the same as pure bulk cocoa butter or cocoa butter dispersed in emulsions stabilized by

510 sodium caseinate or buttermilk powder. Nevertheless, it is the same as in the bulk phase
511 composed of cocoa butter and sunflower lecithin. Additionally to β' -2L crystals, β -3L crystals
512 are present in the droplets. The polymorph is known to modify the crystal hardness (Marangoni,
513 2005), shape at the mesoscopic scale (μm) and aggregation tendency (McClements, 2012).
514 Thus, for the same droplet size, the type of chosen emulsifier and its affinity for the oil phase
515 can modify the crystalline nanostructure of cocoa butter by adding the coexistence with another
516 polymorph. To explain the effect of the sunflower lecithin on the massive partial coalescence
517 occurring in emulsions, it is also worth focusing on the interface. Interfacial tension
518 measurements show that sunflower lecithin behaves as small, low-molecular-weight surfactants
519 (Fig. 7a). Alike surfactant and unlike proteins, phospholipids do not interact with each other at
520 the interface, maintaining a fluid interface. These molecules are also much less efficient than
521 proteins to avoid crystal piercing, due to smaller size and therefore less steric repulsion between
522 drops. Also, adsorption of those molecules onto crystals can change their wettability, increasing
523 their affinity towards water and consequently modifying their positioning at the interface.
524 Indeed AFM shows that the roughness is not increased with lecithin but crystals become visible.
525 Positioning of crystals at the interface or even displacement of crystals in the water phase has
526 been shown by Ergun *et al.* for anhydrous milk fat with sodium dodecyl sulfate (Ergun, Hartel,
527 & Spicer, 2015). This is a bit different from a difference in protrusion and interface roughness
528 between proteins and surfactant observed with anhydrous milk fat and either Tween 20 or
529 sodium caseinate (Thivilliers *et al.*, 2008).

530 To sum up, the dispersed phase is composed of two crystal types, the interface behaves as a
531 surfactant-covered liquid interface also allowing higher water wetting of the crystals. This leads
532 to massive edge piercing between drops likely establishing a continuous network of crystals
533 responsible for the hard and brittle texture (Fig. 9b).

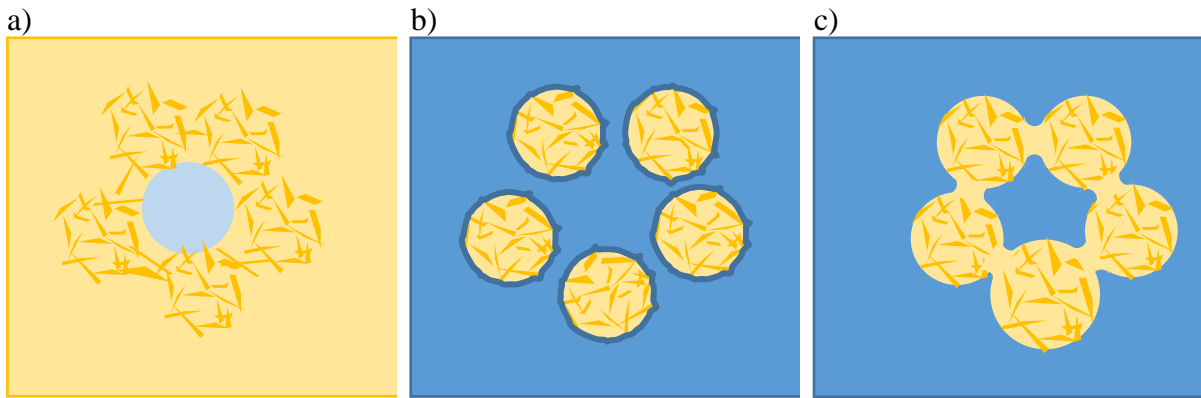
534 4.3 Buttermilk powder

535 The emulsion stabilized by buttermilk powder, composed of both milk proteins and
536 phospholipids, exhibits an interesting spreadability after crystallization at 4°C, meaning neither
537 too hard as with sunflower lecithin, nor too liquid as with sodium caseinate. This texture,
538 appreciable for applications, is due to partial coalescence between droplets as shown by the
539 confocal microscopy pictures giving rise to a water continuous network of connected droplets.
540 The extent of partial coalescence is quite significant, as a large proportion of the droplets belong
541 to finite or percolated clusters, but it does not end by a phase inversion. When this network is
542 heated to complete the freeze-thaw cycle, two phenomena occur: shape relaxation of already
543 connected droplets and possibly additional (temperature-induced) connections, both
544 contributing to the observed macroscopic yellow oil layer at the top of the sample. In the liquid
545 state the cocoa butter has a density of the order of 0.87 g.cm⁻³ meaning that the volume fraction
546 of the initially prepared emulsion is about 38.2 vol% (35 wt%). The layer of oil at the top of the
547 sample represents about 33 vol% to be compared to the initial 38.2 vol%. It therefore seems
548 that 86% of cocoa butter belong to this macroscopic layer, the remaining 14% are dispersed in
549 the water continuous emulsion at the sample bottom (5% melted cocoa butter-in-water whitish
550 emulsion) and scatter the light.

551 At 4°C, the crystal polymorph (β'_2 -2L) in the droplets is the same as in the pure cocoa butter
552 bulk phase as revealed by X-Ray scattering. It is interesting to note that the interface topography
553 is not very different from the lecithin covered one. The protein interface is weakened by the
554 presence of small molecules leading to apparent crystals. It is known that nonionic surfactant
555 may displace milk proteins (Mackie et al., 2003; Woodward et al., 2004) and decrease the
556 elasticity of the interface originating from protein interactions (Derkatch et al., 2007). More
557 recently, it has been shown that Tween 20 exerts the same effect on interfaces covered by silk
558 protein (Qiao, Miller, Schneck, & Sun, 2022, 2024) showing the generality of the phenomenon.
559 In buttermilk powder, the same weakening phenomenon likely occurs and partly explains the
560 presence of crystals at the surface. Nevertheless it should be emphasized that the proteins are
561 not completely removed as the interfacial tension remains at a high level compared to the
562 phospholipid covered interface (sunflower lecithin). The second phenomenon that should be
563 put forward to explain the presence of crystal at the surface is once again the change of crystal
564 wettability induced by the presence of the amphiphilic phospholipids.

565 To sum up, the presence of phospholipids at the interface leads to a weakened layer of proteins
566 and to highly more water wetted crystals visible from the water phase. Significant edge piercing
567 occurs without phase inversion. The existence of a percolated network of connected drops
568 surrounded by an aqueous phase can explain the solid but spreadable texture seen at 4°C after
569 7 days (Fig. 9c).

570



571 Figure 9: Schematic representation of the emulsion structure after crystallization, explaining
 572 the observations for the three different stabilizers a) sunflower lecithin b) sodium caseinate and
 573 c) buttermilk powder.

574

575 In buttermilk powder and lecithin, the presence of low-molecular-weight surfactants allows the
 576 crystals to position at the surface and pierce the interfaces, enabling inter-drop connections.
 577 Such positioning can result from a weakening of the interfacial layer with small molecules
 578 compared to proteins and from adsorption of these small molecules onto crystals rendering them
 579 more water wettable. These crystals are responsible for partial coalescence leading to a gel
 580 texture in the case of buttermilk powder and likely to phase inversion in the case of sunflower
 581 lecithin. In the case of protein stabilized emulsions, the protein film prevents partial coalescence
 582 and the emulsion remains liquid at 4°C and stable through a complete freeze-thaw cycle.

583 This work therefore enabled us to highlight the behaviors, at the two opposite limits, of
 584 highly stabilizing sodium caseinate and highly destabilizing lecithin with regard to
 585 crystallization of the dispersed phase and partial coalescence of emulsions, as well as the

586 intermediate behavior of buttermilk powder. This last stabilizer is therefore the most promising
587 one in order to tune the extent of the phenomenon.

588

589 4. Conclusion

590 The present work reports a study of model emulsions at different observation scales keeping all
591 the parameters constant: droplet size, proportions of cocoa butter and water, and aging time,
592 just varying the type of emulsifiers. This strategy allowed a better understanding of the
593 stabilizer influence on the final texture of crystallizable cocoa butter-in-water emulsions as
594 easily observable even by naked eyes. It was already known that the type of emulsifier could
595 influence the viscoelastic properties of the interfacial film as well as the crystal wetting
596 properties and thus could modify the position of crystals. In the present paper, we show that the
597 sodium caseinate milk protein exhibits a very effective protection against edge piercing
598 annihilating the possibility to induce gelling in cocoa butter-in-water emulsions. In opposition,
599 sunflower lecithin is not protective enough and too massive edge-pricing likely leads to a butter
600 continuous phase. The resulting gel is too hard and brittle for applications. Buttermilk powder,
601 which is composed of both proteins and phospholipids, exhibits an interesting intermediate
602 behavior allowing partial coalescence and leading to spreadable textures without the need to
603 add texturizers that the food industry seeks to remove from products.

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698 SUPPORTING INFORMATION

699 SI 1: Composition of the two fats.

700 SI 2: Composition of sunflower lecithin.

701 SI 3: composition of buttermilk powder.

702 SI 4: Stability of liquid sunflower oil-in-water emulsions stored at 4°C.

703 SI 5: Zooms of AFM pictures highlighting the presence of crystal at the surface in the case of
704 lecithin and buttermilk powder.

705

706 ABBREVIATIONS

707 BP: buttermilk powder

708 CB: cocoa butter

709 CO: copra oil

710 L: sunflower lecithin

711 MCT: middle chain triglycerides

712 P: sodium caseinate protein

713

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