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1 **Drying method determines the structure and the solubility of microfluidized pea**
2 **globulin aggregates**

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11

12 **Abstract**

13 The effects of microfluidization and drying method on the characteristics and techno-
14 functional properties of pea (*Pisum sativum L.*) globulin aggregates were investigated. Pea
15 globulin aggregates were microfluidized at 130 MPa and spray-dried or freeze-dried
16 thereafter. Microfluidization decreased aggregate size and surface hydrophobicity due to
17 protein re-arrangements. Microfluidized pea globulin aggregates showed higher solubility but
18 less suspension stability than non-microfluidized aggregates. Drying favored the re-
19 aggregation of pea globulins with modifications in secondary structure of proteins more
20 marked for spray-drying, decreased surface hydrophobicity and solubility, but increased
21 suspension stability. Spray-dried aggregates were smaller than freeze-dried, with improved
22 suspension stability. These results indicated that microfluidization and drying determine the
23 structure of pea globulin aggregates and their associated techno-functional properties. These
24 findings are crucial for the preparation of plant protein powders in the food industry.

25

26 **Keywords:** Pea globulin, Aggregate, Microfluidization, High dynamic pressure, Spray-
27 drying, Freeze-drying, Microscopy, Structure, Solubility, Suspension stability

28

29 **1. Introduction**

30 The use of vegetal proteins is increasing rapidly in food applications due to their nutritional,
31 environmental and economic benefits. However, their inclusion in food formulation implies
32 adequate techno-functional properties, which mainly depends on the protein structure.
33 Structure and technological properties of vegetal proteins are modified by the technological
34 process applied during extraction (Stone et al., 2015, Fuhrmeister and Meuser, 2003), and
35 conditioning (Sun and Arntfield, 2011, Ghribi et al., 2015, Shen and Tang, 2012). These
36 processes may result in protein denaturation and/or aggregation (Shen and Tang, 2012).

37

38 Generally, globular proteins like whey proteins spontaneously and irreversibly aggregate
39 when heated above 60 °C. The structure of globular protein aggregates formed after heating
40 has been studied in detail (Nicolai and Durand, 2013). Earlier studies showed that in a first
41 step, globular protein participates in a first aggregation or pre-aggregation leading to different
42 structure depending on pH, ionic strength and concentration. Three distinctly morphologies of
43 thermal aggregates have been observed: spherical particles (50 nm-few microns) next to the
44 isoelectric point; strands (< 10 nm width, 10 nm – 10 µm length) far from the isoelectric point
45 or with high net charge; and long semi-flexible fibrils (micron length) during heating at very
46 low pH values (< 2.5) and low ionic strength (Nicolai and Durand, 2013). At higher protein
47 concentration, these aggregates will re-aggregate in a secondary aggregation resulting in
48 larger fractal clusters (Baussay et al., 2004, Mehalebi et al., 2008). Although the general
49 features are common to most globular proteins, pea protein aggregates are widely unknown
50 and need to be characterized for optimal functionality.

51

52 Structure and properties of protein aggregates are modified by technological process such as
53 microfluidization. Microfluidization or dynamic high pressure modifies protein structure due
54 to a combination of turbulence, shear and impact in a liquid by going through a fixed and
55 small geometry. A previous study (Oliete et al., 2018) showed that microfluidization at 70
56 MPa and 130 MPa led to re-arrangements in the structure of pea globulin aggregates resulting
57 in decreased protein particle size and hydrophobicity. Microfluidization at 120 MPa also
58 resulted in modifications in physicochemical and conformational properties of soy protein
59 aggregates that improved protein solubility and emulsifying ability (Shen and Tang, 2012).
60 Dissanayake and Vasiljevic (2009) indicated that microfluidization at 140 MPa improved

61 solubility and emulsifying activity index of whey protein aggregates at pH 7 as a result of the
62 disintegration of insoluble big aggregates into small soluble particles. In these studies,
63 characterization and evaluation of the techno-functional properties of aggregated proteins
64 were performed immediately after microfluidization. However, for industrial use, protein
65 aggregates should follow at least a drying step to facilitate their conservation, transport and
66 utilization.

67

68 Spray-drying and freeze-drying are the two most commonly used methods for drying proteins
69 (Gong et al., 2016). Most of researchers in protein characterization use freeze-drying as a low
70 temperature drying method to preserve the native structure of protein molecules, but the
71 industrial production of food protein involves spray-drying because of the high cost and low
72 productivity of freeze-drying. Differences in temperature and water evaporation process may
73 affect differently protein structure and thus techno-functional properties of proteins (Tang et
74 al., 2009, Mehalebi et al., 2008). The effect of drying method has been studied on the
75 structure and technological properties of legume proteins such as soy protein (Hu et al., 2010),
76 lentil (Joshi et al., 2011), rice dreg (Zhao et al., 2013), chickpea (Ghribi et al., 2015), peanut
77 (Gong et al., 2016), and grass pea (Feyzi et al., 2018) proteins. Freeze-drying legume proteins
78 showed more compact and ordered conformations (Zhao et al., 2013) with higher water/oil
79 holding capacity and lower emulsifying and foaming properties than spray-dried proteins
80 (Joshi et al., 2011, Hu et al., 2010, Ghribi et al., 2015, Gong et al., 2016, Zhao et al., 2013).
81 Authors attributed differences between drying methods to lower protein denaturation during
82 freeze-drying than during spray-drying. However, to our knowledge the effect of the drying
83 method on the characteristics and functionality of plant protein aggregates has not been
84 studied.

85

86 The aim of this work was to determine the effect of microfluidization and drying method
87 (spray-drying vs freeze-drying) on some physicochemical characteristics of pea globulin
88 aggregates accountable for their techno-functional properties. Globulin aggregates were
89 characterized in terms of particle size, microstructure, charge, surface hydrophobicity and
90 secondary structure of proteins. Protein solubility at different pH and suspension stability
91 were also determined.

92

93 **2. Material and Methods**

2.1. Globulin extraction and proximate analysis

Pea flour, provided by Cosucra (Warcoing, Belgium), was a mixture of different cultivars. Flour was defatted by vigorous stirring in 5 volumes of petroleum ether for 1 h at room temperature (twice), and in 5 volumes of ethanol for 1 h at 4 °C (twice). The slurry was vacuum-filtered on a No. 2 porosity glass filter, and dried at room temperature for 48 h. Alkali extraction–isoelectric precipitation was used for globulin extraction as indicated by Messian et al. (2012). The dry flour was poured in a 0.1 M tris-HCl, pH 8 buffer and soluble proteins were extracted for 8 h at 4 °C (ratio flour-to buffer of 1:10). Insoluble materials were removed by centrifugation (10,000 g, 50 min, 20 °C). HCl 0.1 M was added to the supernatant up to pH 4.8 for globulin precipitation. Mixture was stirred for 1 h at 4 °C. Proteins were collected by centrifugation (10,000 g, 25 min, 4 °C), the pellets were washed with distilled water (pellet to water ratio of 1:25). The proteins were collected by centrifugation (10,000 g, 15 min, 4 °C), re-suspended at a concentration of 4 (w/w) and neutralized (pH 7.2) by adding slowly NaOH 0.25 M. The mixture was stirred at room temperature for 1 h, and again centrifuged to remove remaining insoluble material (10,000 g, 15 min, 4 °C). Globulins were frozen for 24 h at -18 °C and then freeze-dried in a Labconco Freeze dryer (Labconco, Kansas city, MO, USA). The process consisted in four steps of 24 h, at -50, -30, 0 and 25 °C. The resulting powder was stored at -18 °C until further use. The dry matter ($95.38 \pm 0.2\%$ (w/w)) was determined according to AOAC International method 923.03 (AOAC, 1990). The protein content was $97.05\% \pm 0.2$ (w/w) on a dry basis as determined by using the Kjeldahl method as AOAC International method 920.87 (AOAC, 1990) with a nitrogen conversion factor of 6.25 (Messian, Sok, Assifaoui, & Saurel, 2013, Shand, Ya, Pietrasik, & Wanasundara, 2007). The mineral content ($2.36\% \pm 0.02$ (w/w)) on a dry basis was determined as AOAC International method 945.39 (AOAC, 1990).

2.2. Preparation of aggregates

Pea protein aggregates were obtained using the protocol described by Chihi, Messian, Sok, & Saurel, (2016). 4% (w/w) globulin dispersions were prepared in 10 mM sodium phosphate buffer, pH 7.2, and stirred overnight at 4 °C to complete hydration. Sodium azide (0.05% w/w) was added as a preservative. The pH was adjusted to 7.2 with 0.1 M NaOH.

The dispersion was centrifuged at 10,000g for 20 min at 20 °C to remove insoluble proteins. The supernatant, with a protein concentration of $3.41\% \pm 0.04$, was poured into tubes (0.5 cm in diameter) and placed in a temperature-controlled water bath previously equilibrated at 40

128 °C, then heated at 1 °C/min from 40 to 90 °C, incubated at 90 °C for 60 min, and rapidly
129 cooled on ice for 10 min. Aggregated pea globulins showed no endothermic peak in the DSC
130 thermogram (data not shown), indicative of complete protein denaturation due to thermal
131 treatment.

132
133 Aggregated protein dispersions were subjected to microfluidization at 130 MPa (denoted as
134 A130) in a LM10 Microfluidizer (Microfluidics, Newton, MA, USA) fitted with a Z-type
135 chamber (G10Z). Samples were passed 3 times through the system. Aggregated protein
136 samples without microfluidization treatment were denoted as A0. A0 and A130 samples were
137 analyzed directly after preparation without drying.

138

139 **2.3.Drying**

140 Spray-drying or freeze-drying method was applied to microfluidized pea globulin aggregates
141 (A130). For spray-drying (A130S) an atomizer Büchi Mini Spray Dryer B-290 (Büchi Sarl,
142 Rungis, France) was used. The outlet temperature was fixed at 65 °C and the inlet temperature
143 to 160 °C during drying. For freeze-drying (A130F) globulin solutions were first frozen for 24
144 h at -18 °C and then freeze-dried in a Labconco Freeze dryer (Labconco, Kansas city, MO,
145 USA). Freeze-drying consisted in four steps of 24 h, at -50, -30, 0 and 25 °C.

146

147 After drying, aggregate samples were equilibrated at constant water activity of $0.293 \pm$
148 0.008% by storing for at least 7 days before analysis in a saturate ambient with potassium
149 acetate. Water activity of aggregates was measured at least in triplicate with an Aqualab
150 Dew Point Water Activity Meter 4TE (Aqua Lab, Pullman, WA, USA). Protein content of
151 dried aggregates was $95.70\% \pm 0.44$ for A130S and $95.42\% \pm 1.82$ determined by the
152 Kjeldahl method as indicated before.

153

154 **2.4.Particle size**

155 Size and distribution of pea globulin aggregates were measured using a laser diffraction
156 particle size analyzer MasterSizer 3000 analyser (Malvern Instruments), with 10 mM
157 phosphate buffer, pH 7.2, 0.05% (w/w) sodium azide as a dispersant. The relative refractive
158 index of the dispersion was taken as 1.09, that is, the ratio of the refractive index of wheat
159 gliadin considered as reference (1.45) to that of the continuous phase (1.33). All
160 determinations were conducted at least in triplicate. Volume-average diameter, $d_{4,3}$ was
161 reported as an indicative measure of particle size [Eq. 1]:

162 $d_{4,3} = \Sigma n_{idi4} / \Sigma n_{idi3}$ [Eq. 1]

163

164 The particle size heterogeneity Span was calculated as [Eq. 2] :

165 $\text{Span} = [(d_{90} - d_{10}) / d_{50}]$ [Eq. 2]

166

167 **2.5. Microscopy**

168 Aggregated pea globulin suspensions were prepared in 10 mM phosphate buffer pH 7.2 for
169 Transmission Electron Microscopy (TEM) by negative staining according to the procedure
170 previously described by Munialo et al. (2014). A 10 μL aliquot of 125mg/mL aggregated pea
171 globulin dispersions was deposited onto a carbon support film on a copper grid. The excess
172 was removed after 30 min using a filter paper (Whatman no. 1, 512-1002, VWR International
173 Europe BVBA, Leuven, Belgium). A droplet of 3% uranyl acetate at pH 3.8 was added for 30
174 s to improve the contrast. Excess uranyl acetate was removed by use of a filter paper. Electron
175 micrographs were obtained using a Hitachi H-7500 transmission electron microscope (Hitachi
176 High-Technologies Europe GmbH) equipped with an AMT camera driven by AMT software
177 (Hitachi) operating at 80 kV.

178

179 **2.6. Fourier transform infrared (FTIR) spectroscopy**

180 The secondary structure of aggregated pea proteins was characterized using FTIR
181 spectroscopy. Aggregated pea protein suspensions (A0 and A130) were poured in a
182 crystallizer forming a thin layer, dehydrated at 40 °C until water elimination and manually
183 ground. A130S and A130F were analyzed as they were. FTIR spectra were collected in the
184 wave number range from 600 to 4000 cm^{-1} with a resolution of 4 cm^{-1} using a Spectrum 65
185 FT-IR spectrometer (Perkin-Elmer, Courtaboeuf, France). The spectra were the average of 32
186 scans.

187 **2.7. Zeta potential**

188 The electrical charge (ζ -potential) of aggregates was measured using a ZetaCompact Z8000
189 zetaphoremeter (CAD Instruments, Les Essarts, Le Roi, France). The samples were diluted
190 with 10 mM phosphate buffer, pH 7.2, to about 25 $\mu\text{g/g}$ to obtain a tracking value of at least
191 100. The temperature of the cell was maintained at ambient temperature. The data was the
192 average value of at least 5 measurements.

193

194 **2.8. Surface hydrophobicity**

195 Protein surface hydrophobicity (H_0) was determined with the fluorescence probe 1-
196 anilinonaphthalene-8-sulfonic acid (ANS; Sigma, St. Louis, MO, USA), according to the
197 method of Kato and Nakai (1980) modified by Karaca et al. (2011). Aggregates were diluted
198 in a range of concentrations from 0.004% to 0.02% (w/w) in 10 mM phosphate buffer pH 7.2.
199 20 μ L of the 8 mM ANS solution prepared in the same buffer were added to 4 mL of each
200 protein sample, and the mixture was kept in the dark for 15 min. To ensure sufficient ANS to
201 link the free surface hydrophobic groups of the protein, a calibration curve was constructed
202 with the most concentrated protein solution (0.02%). The fluorescence intensity was measured
203 using an LS-50B luminescence spectrometer (PerkinElmer, Waltham, MA, USA) at 380 nm
204 (excitation) and 460 nm (emission). Blanks were protein samples without ANS and buffer
205 with ANS. The initial slope of the fluorescence intensity versus protein concentration
206 (mg/mL) plot, calculated by linear regression analysis, was used as an index of protein surface
207 hydrophobicity.

208

209 **2.9.Solubility**

210 1% (w/w) aggregated pea globulin suspensions were hydrated overnight in 10 mM phosphate
211 buffer at pH 3 to 8 at 4 °C. Subsequently, the suspensions were centrifuged at 10000 g for 20
212 min at 4 °C. The protein content in the supernatant was determined by Kjeldhal method.
213 Protein solubility was determined as Nitrogen Solubility Index (NSI) by dividing the nitrogen
214 content of the supernatant by the total nitrogen in the sample (x100%). Analyses were
215 performed in triplicate.

216

217 **2.10. Suspension Stability**

218 The stability of 5% (w/w) aggregated globulin suspensions in 10 mM phosphate buffer pH 7.2
219 was measured by Turbiscan® Lab Expert (Formulation, Toulouse, France). Turbiscan®
220 measurements are based in multiple light scattering. The equipment possesses two optical
221 sensors that measure the transmitted and the backscattered near-infrared monochromatic light
222 ($\lambda = 800\text{nm}$). Mengual, Meunier, Cayre, Puech, & Snabre (1999) indicated the ability of this
223 technique to characterize particle or aggregate size variation and particle/aggregate migration.
224 In diluted systems (transmission variation $> 0.2\%$), these phenomena are measured by the
225 transmission light intensity of the sample as a function of sample height and time. The
226 measurement of the backscattering light in non-opaque systems can give erroneous
227 information since the backscattering profiles can be affected by the partial reflection of the
228 light crossing the sample. Suspensions were maintained under agitation until analysis.

229 Samples were placed in flat-bottomed cylindrical borosilicate glass tubes and introduced into
230 Turbiscan® measurement cell. Turbiscan detection head scanned the sample by moving
231 vertically along the analysis cell and acquiring data every 40 µm. The light scattering profiles
232 were recorded every 10 minutes for 12 hours. Differences in the transmission profiles along
233 time indicate sample instability. Light transmitted intensity (T) is affected by the average
234 diameter of particles and the mean free-path of photon. T may change over time and height.
235 Light transmitted intensity (T) Based on Turbiscan® graphs three different regions were
236 distinguished along the tube axis: the bottom, the middle and the top. Variations of T in the
237 top and in the bottom of the sample are related to migration phenomena, such as clarification
238 (top) and sedimentation (bottom). The variations of T in the middle part of the sample
239 indicate the interaction phenomena between particles, and thus particle size variation. Based
240 on the obtained spectra, suspension stability can be calculated by Turbiscan stability index
241 (TSI) according to equation Eq.3

$$242 \quad TSI = \sum_i \frac{\sum_h |Scan_i(h) - Scan_{i-1}(h)|}{H} \quad [Eq.3]$$

243 Where H: sample height from bottom of the cell to the meniscus

244 $Scan_i(h)$: intensity of scanning light (time is i, height is h)

245 $Scan_{i-1}(h)$: intensity of scanning light (time is i-1, height is h)

246 The lowest TSI values indicated the most stable suspensions. Turbiscan® spectra were
247 analyzed with the software Turbisoft-Lab version 2.2.0.82-5 (Formulation, Toulouse,
248 France).

249

250 **2.11. Statistical analysis**

251 Differences between samples (A0, A130, A130S, A130F) were studied by analysis of
252 variance (one-way ANOVA). Significance was set at $p < 0.05$. Tukey's post-hoc least-
253 significant-differences method was used to describe means with 95% confidence intervals.
254 The statistical analyses were performed using Statistica software, version 12 (Tulsa, OK,
255 USA).

256

257 **3. Results**

258 **3.1. Particle size**

259 The particle size distribution of pea globulin aggregates obtained by laser granulometry at pH
260 7.2 was displayed in Figure 1. Particle size distribution of pea aggregate samples before
261 microfluidization and drying (A0) showed a high peak around 600 nm that corresponded to

262 the highest volume fraction of aggregates. Another heterogenic population with particle size
263 ranging from ~4 to ~200 μm was observed and corresponded to highly aggregated pea
264 globulins. When microfluidization at 130 MPa was applied (A130) particle size distribution
265 shifted to smaller values compared to A0 (Figure 1), decreasing the volume-average diameter
266 and the distribution heterogeneity (Table 1). Drying microfluidized pea globulin aggregates
267 (A130S, A130F) caused the drastic reduction of the smallest-sized population and formed big-
268 sized aggregates (1 – 100 μm) (Figure 1). Freeze-drying (A130F) showed higher $d_{4,3}$ values
269 compared to A130S (Table 1).

270

271 Figure 1.

272

273 Table 1.

274

275 **3.2. Microscopy**

276 Figure 2 presented transmission electron micrographs (TEM) of pea globulin aggregates at pH
277 7.2. Pea globulin aggregates before microfluidisation and drying (A0) appeared as small
278 curved strands, which agglomerated and formed secondary aggregates (Figure 2a). After
279 microfluidization at 130 MPa (A130), secondary aggregates disappeared and primary
280 aggregates showed smaller and more angular-shaped than in A0 (Figure 2b). Drying after
281 microfluidization (A130S, A130F) favored the formation of voluminous secondary aggregates
282 compared to A130, especially when freeze-drying (A130F) was used compared to spray-
283 drying (A130S) (Figure 2 c and d respectively).

284

285 Figure 2.

286

287 **3.3. FTIR spectra analysis**

288 Figure 3 showed the amide I region (1700 – 1600 cm^{-1}) of pea protein aggregates, permitting
289 to identify the characteristic bands of the protein secondary structure. Amide I region mainly
290 comprises the stretching vibration of C=O (Ellepola, Choi, and Ma, 2005). The obtained
291 bands were assigned to different conformations according to existing data (Yang, Liu, Zeng,
292 and Chen, 2018, Gharsallaoui et al., 2012) as indicated in Table 2. Microfluidization at 130
293 MPa (A130) decreased the intensity of peaks 1602 cm^{-1} (corresponding to vibration of amino
294 acid residues), 1614 cm^{-1} (corresponding to antiparallel β -sheet), 1634 cm^{-1} , 1641 cm^{-1}

295 (corresponding to β -sheet) and 1694 cm^{-1} (corresponding to aggregated strands), compared to
296 A0, whereas it increased the intensity of peaks 1668 cm^{-1} and 1681 cm^{-1} (corresponding to β -
297 turn). Drying microfluidized pea aggregates by spray-drying (A130S) markedly increased the
298 intensity of peak 1652 cm^{-1} (corresponding to random coil), 1683 cm^{-1} (corresponding to β -
299 turn), and 1695 cm^{-1} (corresponding to aggregated strands) compared to A130. In parallel,
300 freeze-drying slightly increased the intensity of peaks 1630 cm^{-1} and 1638 cm^{-1}
301 (corresponding to β -sheet), and reduced the intensity of peaks 1646 cm^{-1} (corresponding to
302 random coil), and 1662 cm^{-1} and 1684 cm^{-1} (corresponding to β -turn).

303

304 Figure 3 and Table 2

305

306 **3.4. Surface hydrophobicity and ζ -potential**

307 The surface hydrophobicity (H_o) of aggregates at pH 7.2 was evaluated using an ANS
308 fluorescence probe (Table 1). Pea globulin aggregates before microfluidization and drying
309 (A0) showed the highest H_o values. Microfluidization at 130 MPa (A130) significantly
310 decreased surface hydrophobicity of pea globulin aggregates (~6% reduction). Drying after
311 microfluidization (A130S, A130F) notably decreased H_o (~30% reduction), but no significant
312 differences were observed between freeze- (A130F) and spray-drying (A130S) methods.

313

314 No significant differences were observed in the charge (ζ -potential) of pea globulin
315 aggregates due to the technological process i.e. microfluidization and drying (Table 1).

316

317 **3.5. Protein Solubility**

318 The solubility profile of pea globulin aggregates as a function of pH (Figure 4) showed an
319 inverted bell shape with the minimum around pH 5 which is near the isoelectric point (pI) of
320 globulins. Microfluidization at 130 MPa did not significantly improved solubility of pea
321 globulin aggregates compared to A0. Drying microfluidized aggregates (A130S, A130F)
322 notably reduced solubility of microfluidized pea globulin aggregates except at pH 5.
323 Comparing drying methods, no significant differences were observed between freeze-dried
324 samples (A130F) and spray-dried samples (A130S).

325

326 Figure 4.

327

328 **3.6. Suspension stability**

329 Figure 5 showed the transmission profiles of pea aggregates obtained by Turbiscan® in delta
330 mode, i.e. the first scan was subtracted from all other scans during time, which allowed easier
331 visualization of the transmission flux variations (Cvek, Mrlik, Moucka, and Sedlacik, 2018).

332
333 A0 samples showed a stable transmission profile (Figure 5a). The decrease of transmission in
334 the bottom of the sample indicating modest sedimentation of particles in suspension would be
335 negligible since transmission variation did not reach 2%. In A130 samples, T noticeably
336 increased in the top of A130 sample (Figure 5b) indicating the formation of a clarification
337 layer. The increase in the middle of the sample indicated that particles increased in size
338 (flocculation) allowing photons to go through the sample without being diffused.

339
340 Drying of microfluidized pea globulin aggregates (A130S, A130F) induced less transmission
341 flux variation than A130 samples (Figure 5c and d respectively). A130S formed a limited
342 clarification layer at the top of the sample (Figure 5c). A130F formed a clarification layer
343 similar to A130 but the increase in particle size (transmission increase in the middle of the
344 sample) was smaller (Figure 5d).

345
346 Figure 5.

347
348 Figure 6 permitted the analysis of the global stability by means of TSI variation. A0 showed
349 the smallest TSI variation in accordance with the most stable transmission profile. A130
350 showed the highest TSI values as a result of the clarification and flocculation phenomena that
351 took place during measurements. Instability in A130 was especially intense during the first
352 five hours as indicated the notable TSI variation. Drying decreased the instability of samples
353 compared to A130, especially in A130S.

354
355 Figure 6.

356 357 **4. Discussion**

358 *Microfluidization effect on pea globulin aggregates characteristics*

359 Pea globulin aggregates before microfluidization and drying (A0) appeared as small curved
360 strands in the micrographs obtained by TEM, as already indicated by Nicolai et al. (2011) and
361 Jung et al. (2008) for β -lactoglobulin aggregates at neutral pH. Primary aggregates
362 agglomerated into secondary aggregates as fractal clusters in which a self-similar structure

363 was repeated (Baussay et al., 2004, Mehalebi et al., 2008). β -sheet were the dominant
364 secondary structure in A0 as revealed the strong absorption at 1640 cm^{-1} in the amine I region,
365 confirming previous results concerning legume globulins: soy protein thermal aggregates
366 (Zhang, Liang, Tian, Chen, and Subirade, 2012), faba bean protein isolate (Yang et al., 2018),
367 and field pea and kidney bean globulins (Shevkani, Singh, Kaur, and Rana, 2015). The
368 negative charge obtained in all pea aggregate samples was related to the working pH value
369 (pH 7.2) which is higher than pI of vicilins (pI 4.5, Ezpeleta et al., 1996) and pI of legumin
370 acid subunit (pI 4.7, Krishina et al., 1979). At pH 7.2, amine and carboxylic free groups of
371 proteins are deprotonated. Charge values were included in the range between -10 and -15mV
372 defined by Riddick (1968) as aggregation threshold, which is in agreement with the
373 observation of protein aggregation.

374

375 Microfluidization at 130 MPa modified the structure of pea globulin aggregates as clearly
376 observed by TEM, FTIR, and particle size distribution. The combination of turbulence, shear
377 and collisions caused by microfluidization at 130 MPa broke secondary aggregates and
378 modified the conformation of primary aggregates which appeared smaller, more
379 homogeneous in size and more angular in shape than in A0. Small sized aggregates obtained
380 by microfluidization (compared to initially present aggregates) underwent structural re-
381 arrangement. Microfluidization seemed to induce the unfolding of β -sheet structures
382 producing a self-reassembly to less ordered structures as β -turns, while most of the other
383 secondary structures of pea protein aggregates were almost maintained. Yang et al. (2018)
384 also indicated that high pressure homogenization at 207 MPa had a certain impact on faba
385 bean protein, but most of the secondary structures were intact. These authors however
386 observed a decrease of α -helix and β -turn after homogenization. Wang, Li, Jiang, Qi and
387 Zhou, (2014) signaled that the processing conditions such as temperature and time determine
388 the protein secondary structures of soybean proteins. New re-arrangements hid hydrophobic
389 groups, made them less accessible to the binding of ANS fluorescence probe, and resulted in
390 decrease of surface hydrophobicity (Oliete et al., 2018). However, Shen and Tang (2012)
391 observed an increase in hydrophobicity after 120 MPa microfluidization of soy aggregates
392 applied after a 30 min thermal treatment. Hydrophobicity behavior of aggregates was clearly
393 dependent on the temperature and time used in the thermal aggregation (Shen and Tang, 2012,
394 Wang et al., 2014). The charge of pea globulin aggregates was not significantly modified by
395 microfluidization as already indicated in previous works (Oliete et al., 2018). The absence of
396 significant differences in the ζ -potential within samples could be related to the presence of

397 salts coming from the extraction method (~2.4% mineral content in the dried protein isolate).
398 Salt could surround the protein, reducing the ζ - potential absolute value by screening effect
399 and thus the amount of electrostatic repulsive forces (Lam et al., 2018).

400

401 Structural changes induced by microfluidization at 130 MPa did not reach to significantly
402 improve solubility of pea globulin aggregates. Solubility is an important prerequisite for
403 proteins to be used in food applications, since it affects all other techno-functional properties.
404 Dissanayake and Vasiljevic (2009) and Shen and Tang (2012) indicated that microfluidization
405 of whey and soy aggregates increased solubility, as a result of several complementary factors.
406 First of all, high shearing due to microfluidization will disrupt large insoluble aggregates into
407 small soluble ones (Shen and Tang, 2012), and size reduction would decrease sedimentability
408 of particles (Meerdink and Van't Riet, 1995). In the case of pea globulin aggregates,
409 microfluidization at 130 MPa decreased the volume-average diameter of particles. However,
410 the non-negligible residual big sized particles in A130 would hinder the increase of solubility
411 caused by the increased in submicron sized particles. These big sized particles (>10 μm)
412 could come from the non-microfluidized sample or could be formed after microfluidization.
413 New arrangements resulting in less ordered secondary structure could favour interactions
414 between particles. Secondly, protein solubility depends on the protein-water interactions
415 which are driven mainly by the protein surface charge and the presence of free hydrophobic
416 groups, at pH values far from the pI. Since no difference in particle charge was observed
417 between A0 and A130, we rather suggest that hydrophobic attractive interactions play a
418 predominant role regarding hydration properties of protein aggregates. In fact, Karaca et al.
419 (2011) observed a negative correlation between protein solubility and surface hydrophobicity.
420 Lower surface hydrophobicity made microfluidized aggregates less compact and more
421 flexible (Tang et al., 2009) than non-microfluidized aggregates. Lower aggregate compactness
422 would facilitate the interactions between proteins and water molecules (Feyzi et al., 2018). In
423 the present case, similar solubility values between microfluidized and non-microfluidized pea
424 globulin aggregates indicated that, despite significant difference in hydrophobicity, the
425 presence of big sized particles would define the solubility behavior of pea globulin
426 aggregates. Microfluidization at 130 MPa was in detrimental to suspension stability as
427 revealed by enhanced flocculation and clarification phenomena during Turbiscan® test . New
428 re-arrangements induced by microfluidization would favour protein-protein interactions,
429 resulting in increasing particle size along time, which was not measurable by laser diffraction
430 due to highly diluted sample conditions. These interactions were probably driven by hydrogen

431 bonds. Electrostatic interactions are managed by particle charge which did not significantly
432 vary between samples in the present work. Hydrophobic interactions decreased between A0
433 and A130 as was indicated by the significant reduction of surface hydrophobicity measured
434 by fluorescence probing.

435

436 *Drying effects on microfluidized pea globulin aggregates characteristics*

437 Drying after microfluidization (A130F, A130S) caused the re-aggregation of pea globulin
438 proteins, as indicated by the particle size data and the TEM observations. Drying increased
439 connection points between primary aggregates leading to branched fractal objects. These
440 effects were more noticeable when freeze-drying (A130F) was used compared to spray-drying
441 (A130S). Other authors (Cepeda et al., 1998; Ghribi et al., 2015; Joshi et al., 2011; Zhao et
442 al., 2013) already indicated that freeze-dried protein showed higher particle size than other
443 more intense drying methods such as spray-drying or hot air convection. In the case of freeze-
444 drying, local cryo-concentration during freezing would increase opportunities of aggregated
445 proteins to come into close contact with one another. In consequence freeze-drying would
446 favour molecular interactions such as electrostatic interactions, hydrophobic interactions and
447 even disulfide bridges (Gong et al., 2016) resulting in compact protein conformations (Zhao et
448 al., 2013). On the other hand, spray-drying process forces the sample through a nozzle
449 forming small droplets that encounter dry and hot air. The drastic kinetics conditions of spray-
450 drying process compared to freeze-drying may limit interactions between molecules and make
451 aggregation more difficult. Differences between drying methods have been observed even in
452 the secondary structure of pea proteins as other authors (Feyzi et al., 2018; Lan, Xu, Ohm,
453 Chen and Rao, 2018). Peaks in the amide I region from dried samples shifted compared to
454 peaks of non-dried samples, suggesting that the protein structures were affected by the drying
455 methods (Zhao et al., 2013). Freeze-drying (A130F) seemed to increase the β -sheet structure
456 and decreased the random coils and β -turns. Spray-drying (A130S) markedly increased
457 random coils which became the predominant structure, β -turns and aggregated
458 strands indicating significant conformation changes to less ordered secondary structure.
459 Simultaneously, the drying method did not show a clear effect on H_o . Similar values of H_o for
460 A130S and 130F samples were indeed observed despite different effects of the drying method
461 on secondary structure of proteins. Thus, new arrangements between molecules in protein
462 aggregates induced by dehydration (A130S, A130F), tended to bury polypeptides with
463 hydrophobic groups which were initially exposed in non-dried microfluidized pea globulin
464 aggregates, decreasing surface hydrophobicity. Whilst Zhao et al. (2013) signaled higher

465 surface hydrophobicity in spray-drying rice dreg protein isolate compared to freeze-dried,
466 Gong et al. (2016) observed higher H_o values in freeze-dried peanut isolate than in spray-dried
467 one. Feyzi et al. (2018) signaled different effect of drying method on H_o depending on the
468 protein extraction method used.

469

470 Changes in the structure of microfluidized pea globulin aggregates caused by drying
471 decreased protein solubility but improved suspension stability, compared to their non-dried
472 counterpart. The decrease in solubility of dried microfluidized pea globulin aggregates was
473 related to higher particle size and thus higher sedimentability of rehydrated aggregates
474 (A130S, A130F) compared to non-dried microfluidized aggregates (A130). The negative
475 effect of increasing particle size due to drying on protein solubility seemed to prevail over the
476 positive effect caused by the decrease of the surface hydrophobicity resulting from re-
477 aggregation. This statement was already suggested above to explain the effect of
478 microfluidization on pea globulin aggregate solubility. The effect of particle size on pea
479 globulin solubility behavior overcomes the effect caused by re-arrangement in aggregate
480 structure modification. Comparing drying methods, freeze-drying (A130F) and spray-drying
481 (A130S) did not show significant differences in solubility. Contradictory results have been
482 published about the effect of the drying method on protein solubility. Hu et al. (2010) and
483 Sumner et al. (1981) reported that spray-dried soy and pea proteins had lower solubility
484 compared to the freeze-dried counterparts. However, Cepeda et al. (1998), Johsi et al. (2011),
485 and Zhao et al. (2013) reported that the solubility of spray-dried faba bean, soy and rice dreg
486 proteins respectively was higher than that of freeze-dried samples. In the case of
487 microfluidized pea globulin aggregates, the presence of big sized particles in both spray- and
488 freeze-dried aggregates would determine their solubility behavior. Drying A130S and A130F
489 improved suspension stability compared to A130. Protein re-aggregation caused by drying
490 would create a stable structure with limited particle size evolution, probably due to enhanced
491 steric repulsion. Increase in protein particle interactions in A130S and A130F was hindered
492 compared to A130, and led to a reduction of the clarification effect. Freeze-dried
493 microfluidized pea globulin aggregates (A130F) showed lower suspension stability than
494 A130S which was in accordance with higher initial particle size.

495

496 **5. Conclusion**

497 In the current study, the effect of microfluidization and drying method on the structure,
498 solubility and suspension stability of pea globulin aggregates were investigated.

499 Microfluidization at 130 MPa broke pea globulin aggregates and formed smaller, more
500 angular-shaped, and lower surface-hydrophobicity particles with less ordered protein structure
501 compared to pea globulin aggregates before microfluidization. Solubility of pea globulin
502 aggregates seemed to be defined by particle size more than protein structure. However, the
503 new re-arrangements produced by microfluidization led to less suspension stability due to
504 time-evolving flocculation. Drying microfluidized aggregates favored re-aggregation of
505 particles with modifications in structural conformation of proteins, and significantly decreased
506 surface hydrophobicity. The new structures created in dried microfluidized pea globulin
507 aggregates decreased solubility but increased suspension stability. Comparing drying methods
508 spray-dried aggregates showed lower re-aggregation than freeze-dried aggregates, resulting in
509 smaller particle size what improved suspension stability. This study showed that drying
510 methods determine the structure of microfluidized pea globulin aggregates and will impact
511 their techno-functional properties. These findings are decisive for using pea globulin
512 aggregates as dry powders in the preparation of suspensions, emulsions and gels for the food
513 industry.

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521

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Figure captions

Figure 1. Particle size distribution profiles of pea globulin aggregates before microfluidization and drying (A0), after microfluidization at 130 MPa (A130), and after microfluidization and spray-drying (A130S) or freeze-drying (A130F) in 10 mM phosphate buffer at pH 7.2.

Figure 2. Transmission electron micrographs (TEM) of pea globulin aggregates before microfluidization and drying (A0) (a), after microfluidization at 130 MPa (A130) (b), and after microfluidization and spray-drying (A130S) (c) or freeze-drying (A130F) (d) in 10 mM phosphate buffer at pH 7.2.

Figure 3. Deconvoluted FTIR spectra in the amine I region of pea globulin aggregates before microfluidization and drying (A0), after microfluidization at 130 MPa (A130), and after microfluidization and spray-drying (A130S) or freeze-drying (A130F).

Figure 4. Nitrogen Solubility Index (%) of pea globulin aggregates before microfluidization and drying (A0), after microfluidization at 130 MPa (A130), and after microfluidization and spray-drying (A130S) or freeze-drying (A130F).

Figure 5. Turbiscan® spectra of pea globulin aggregates before microfluidization and drying (A0), after microfluidization at 130 MPa (A130), and after microfluidization and spray-drying (A130S) or freeze-drying (A130F) in 10 mM phosphate buffer at pH 7.2.

Figure 6. Turbiscan Stability Index (TSI) variation of pea globulin aggregates before microfluidization and drying (A0), after microfluidization at 130 MPa (A130), and after microfluidization and spray-drying (A130S) or freeze-drying (A130F) in 10 mM phosphate buffer at pH 7.2.

Figure 1.

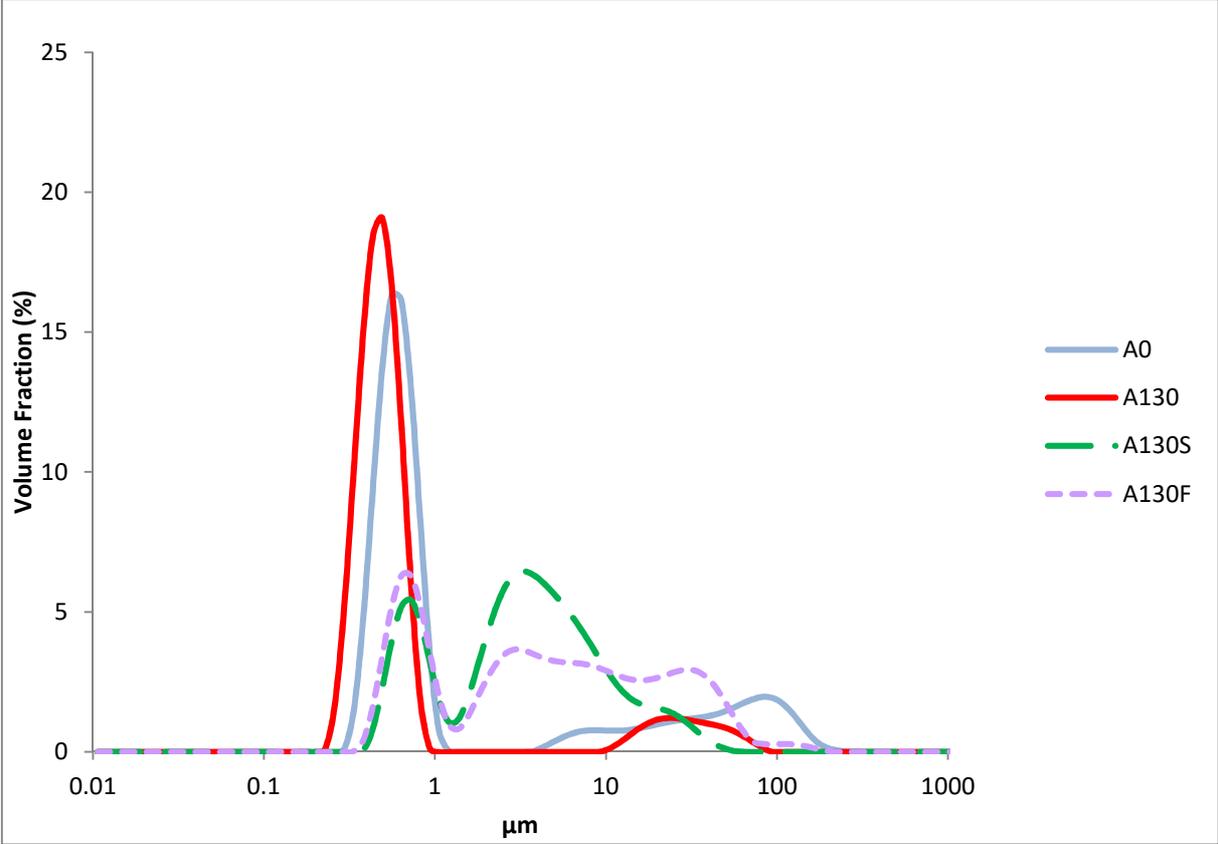


Figure 2.

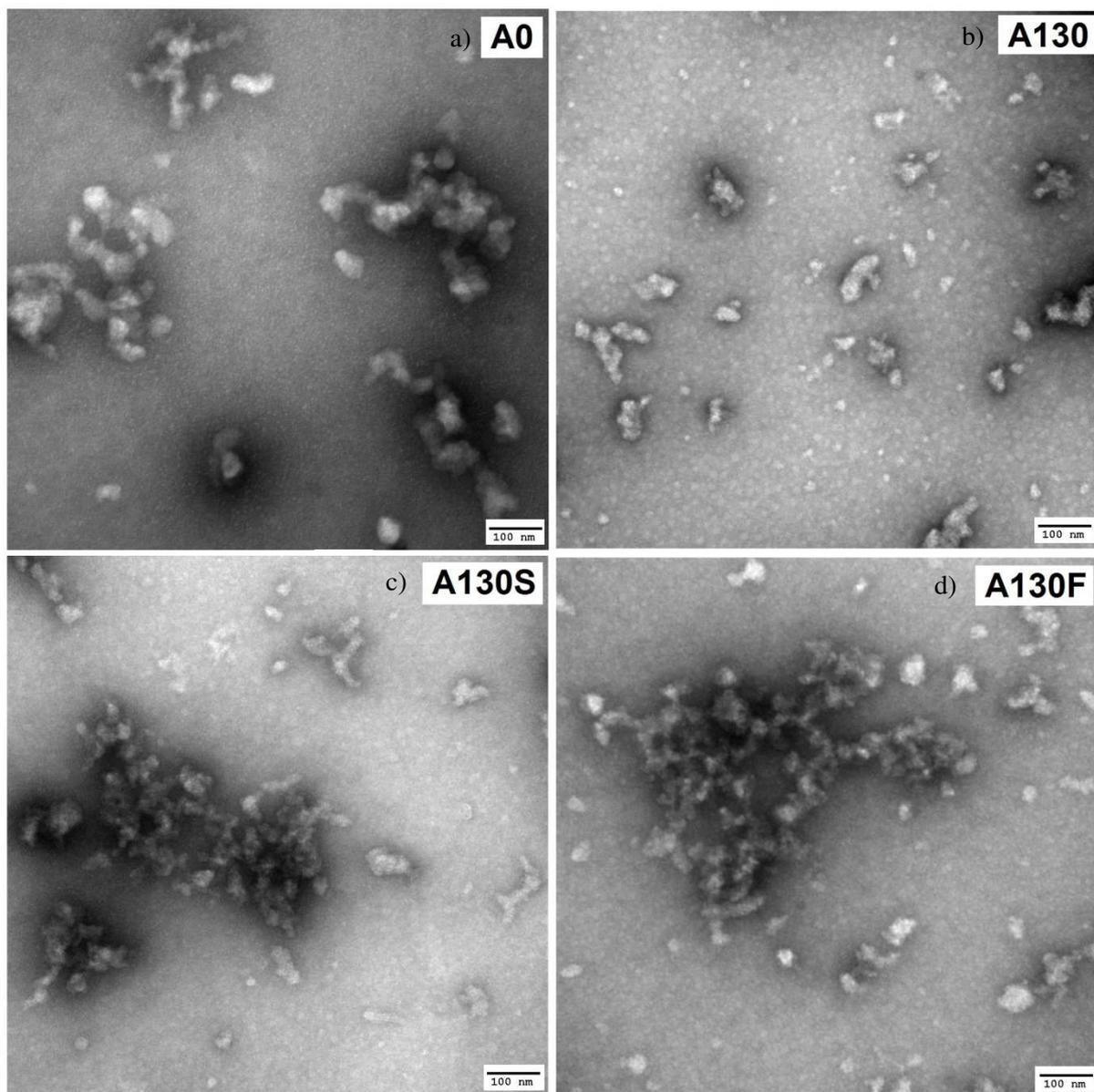


Figure 3

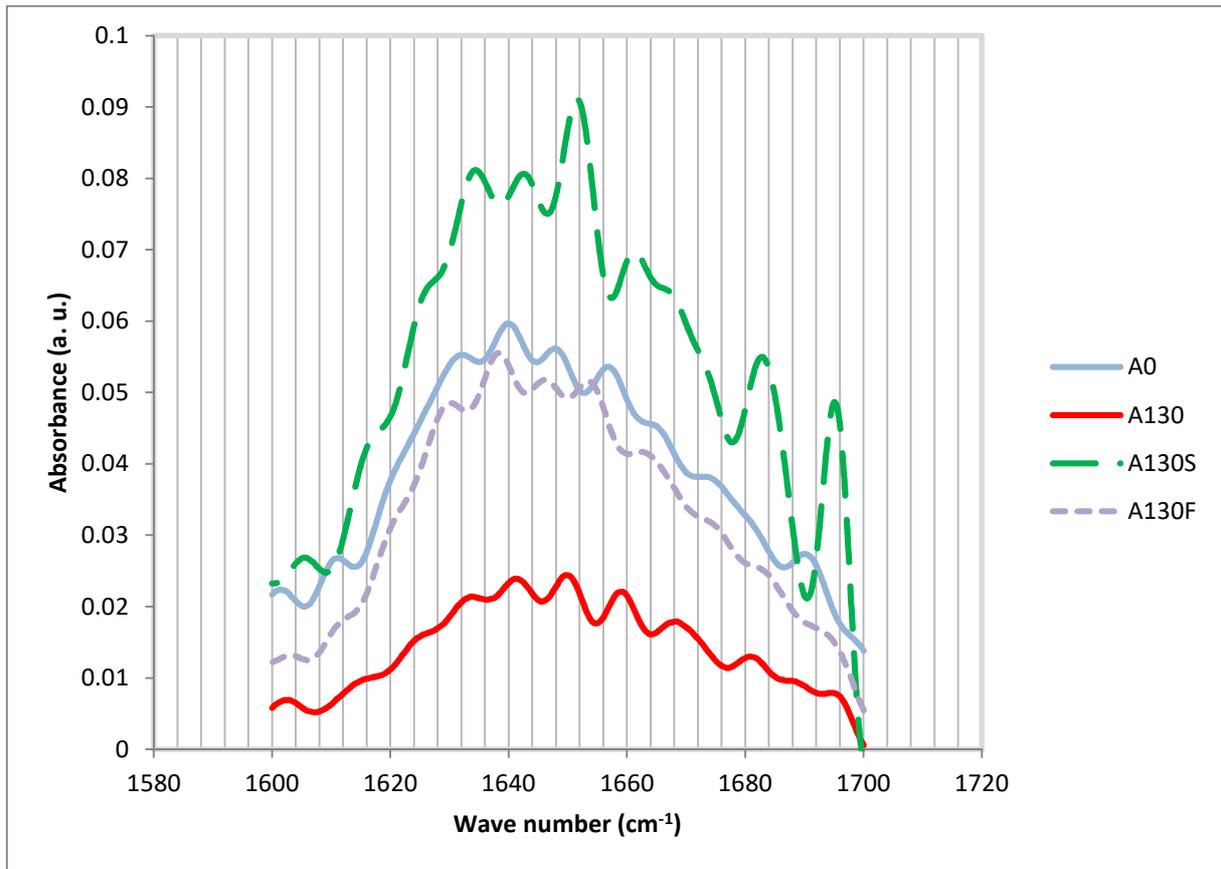


Figure 4.

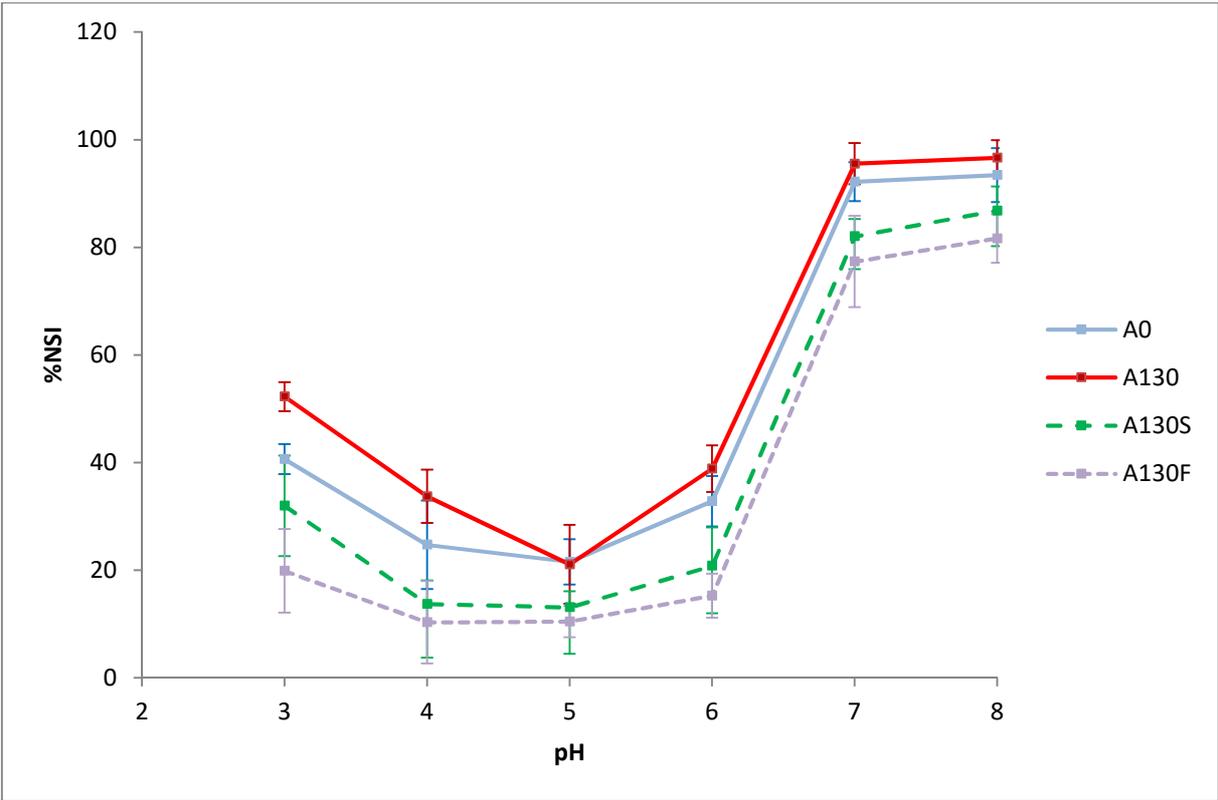
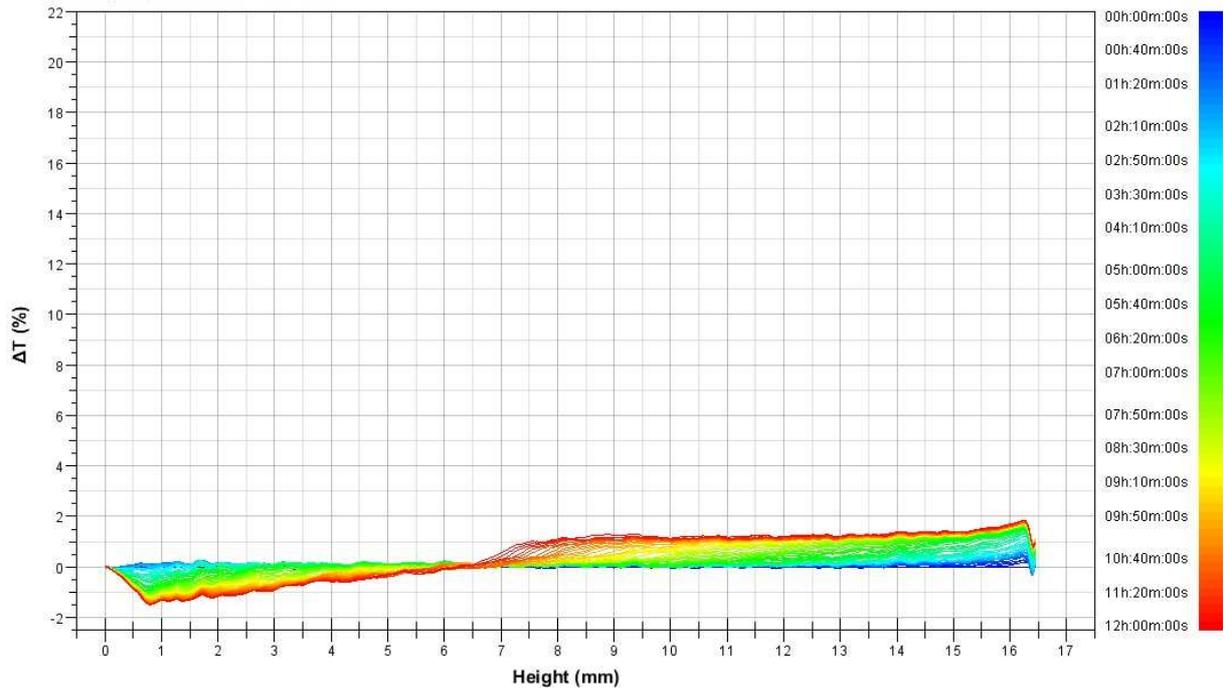
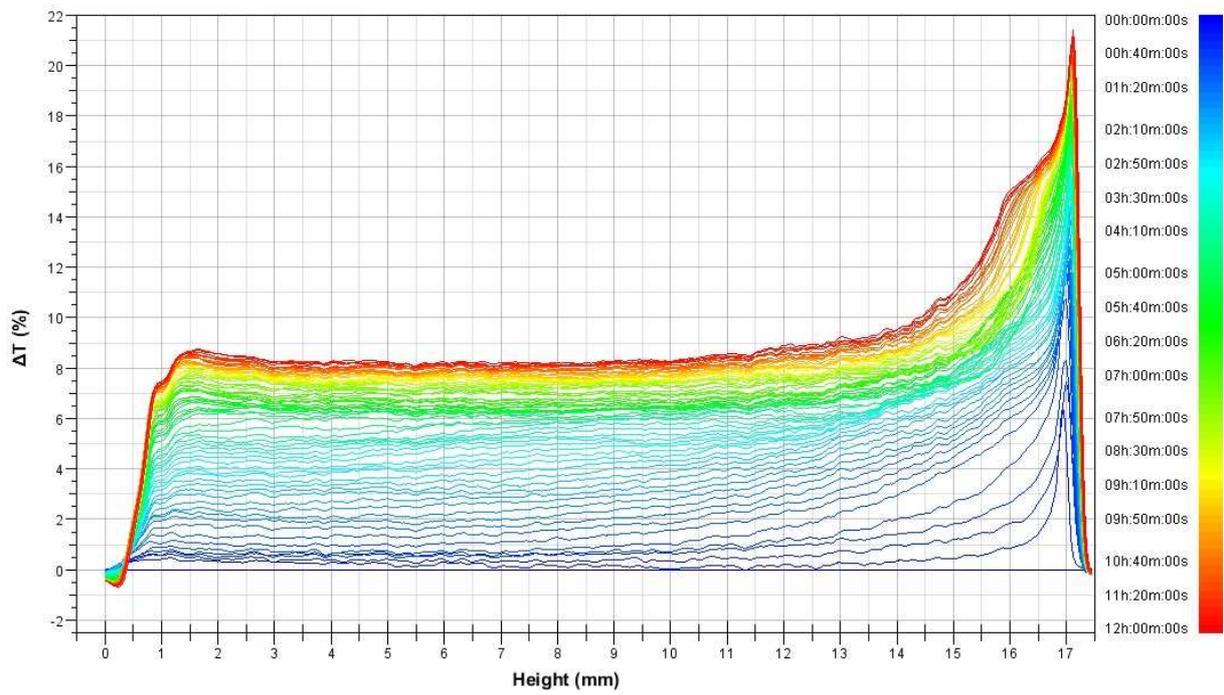


Figure 5.

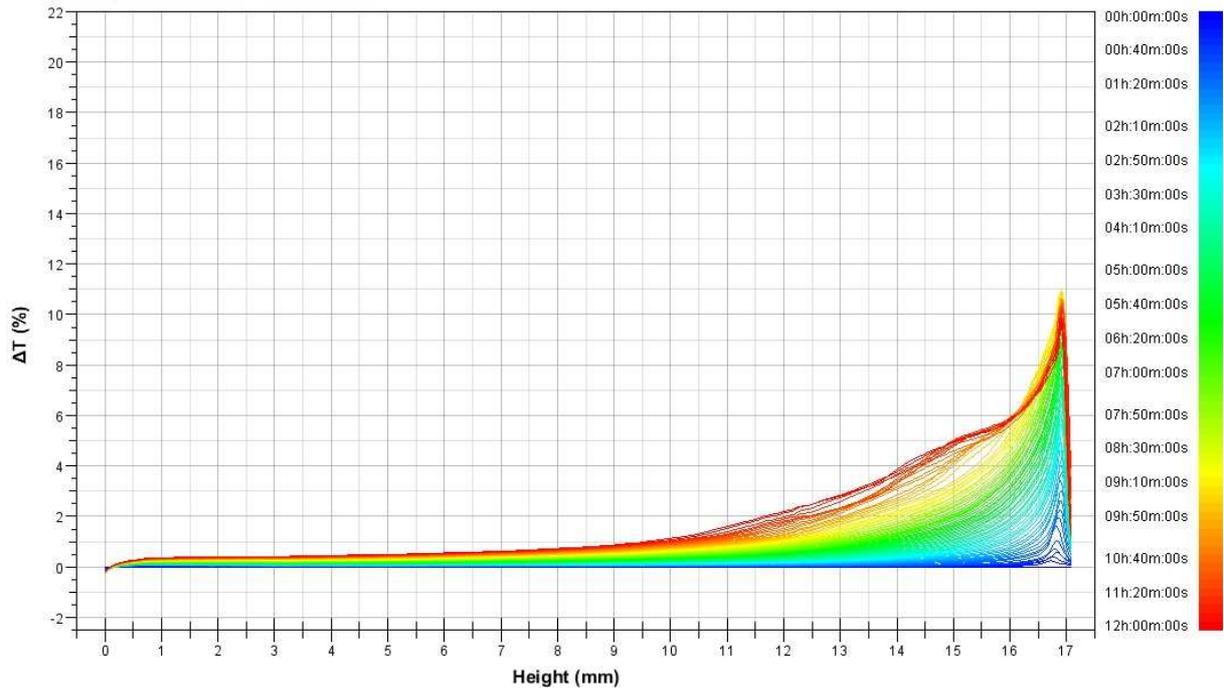
a) A0



b) A130



c) A130S



d) A130F

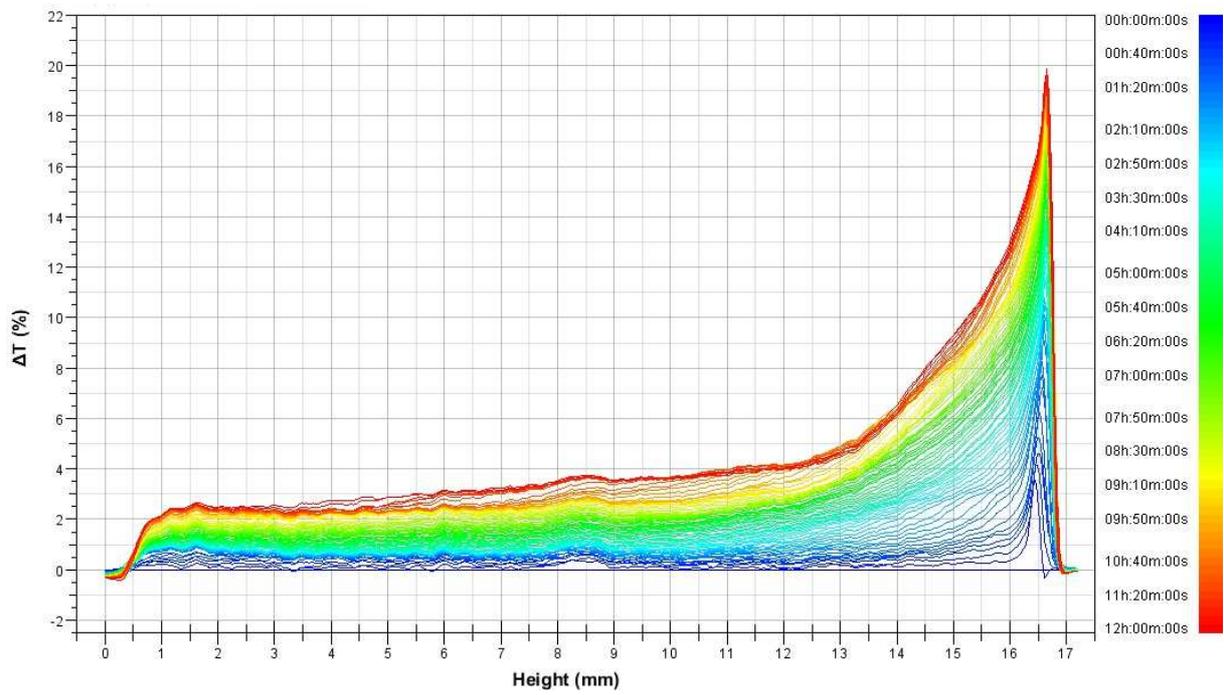
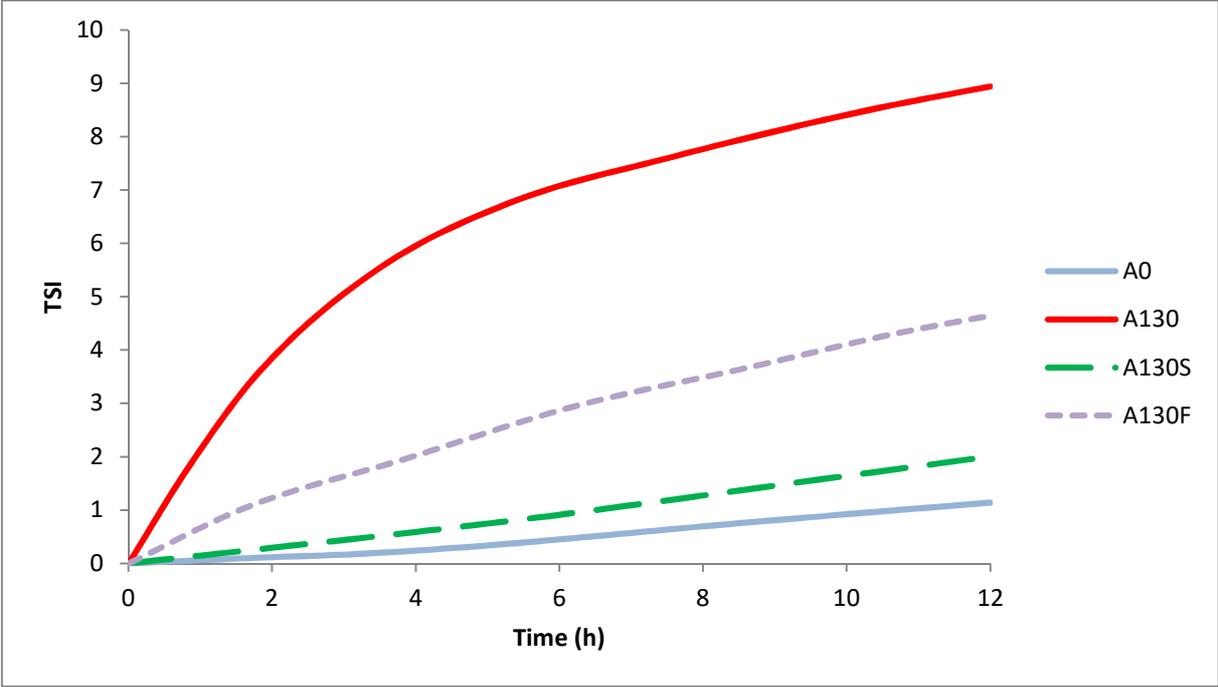


Figure 6.



Tables

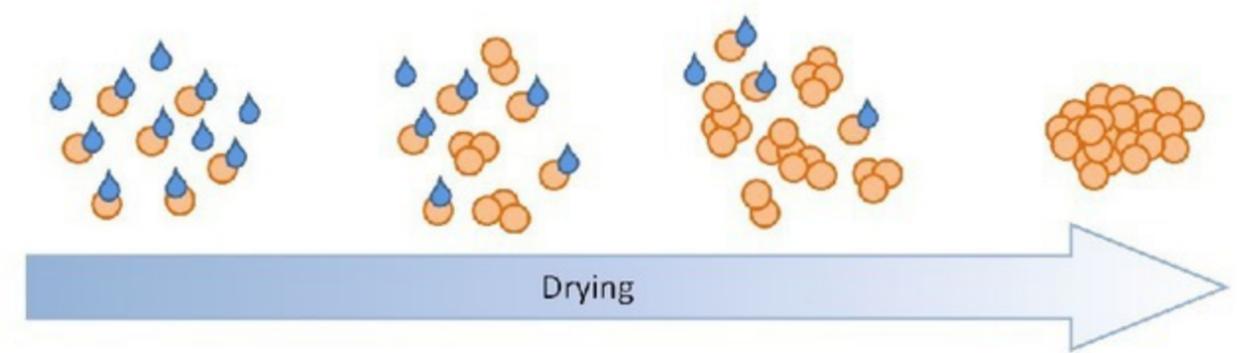
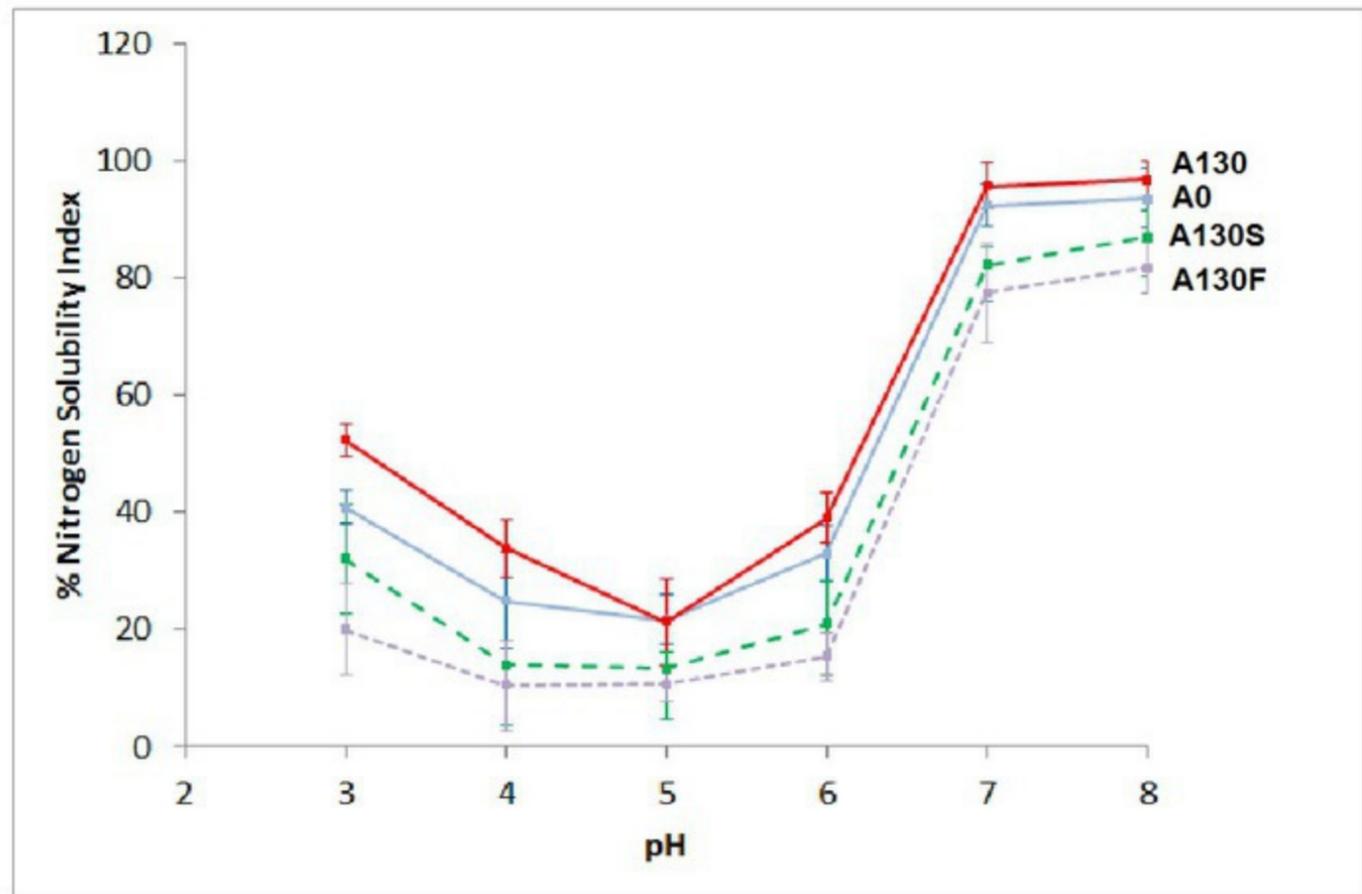
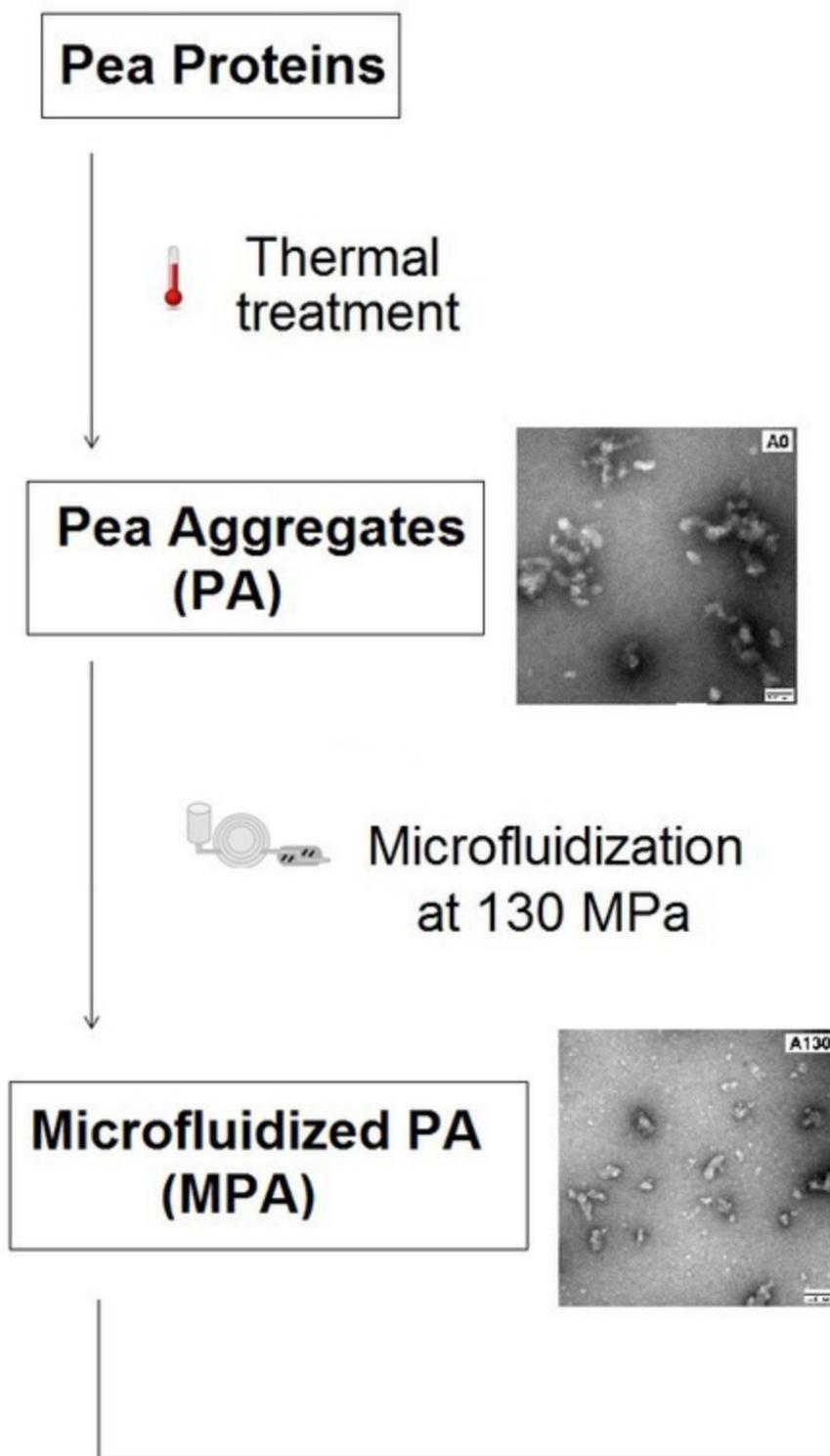
Table 1. Particle size distribution in terms of volume-average diameter ($d_{4,3}$) and heterogeneity (Span), surface hydrophobicity (H_o), and charge (ζ -potential) of pea globulin aggregates before microfluidization and drying (A0), after microfluidization at 130 MPa (A130), and after microfluidization and spray-drying (A130S) or freeze-drying (A130F) in 10 mM phosphate buffer at pH 7.2.

	$d_{4,3}$	Span	H_o	ζ -potential (mV)
A0	15.23 ± 2.37 c	95.48 ± 13.62 c	6007.00 ± 104.25 c	-13.53 ± 0.42 a
A130	4.11 ± 0.13 a	31.39 ± 1.36 b	5618.33 ± 70.57 b	-13.35 ± 0.87 a
A130S	5.59 ± 0.46 a	3.74 ± 0.24 a	3880.00 ± 18.03 a	-13.40 ± 0.94 a
A130F	11.90 ± 1.55 b	8.11 ± 0.60 a	3954.33 ± 18.72 a	-13.38 ± 0.51 a

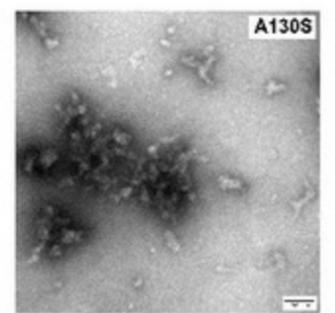
Different superscripts in the same column represent significant differences among different samples (Tukey's *post hoc* test).

Table 2. Assignment of de-convoluted amide I bands in the FTIR spectrum of pea globulin aggregates before microfluidization and drying (A0), after microfluidization at 130 MPa (A130), and after microfluidization and spray-drying (A130S) or freeze-drying (A130F) in 10 mM phosphate buffer at pH 7.2

Assignment	Wavenumber (cm^{-1})			
	A0	A130	A130S	A130F
Vibration of amino acid residues	1602	1602	1605	1603
Anti-parallel β -sheet	1611	1614	1616	1612
Intermolecular hydrogen bonds in β -sheet	1622	1624	1625	1622
β -sheet	1632	1634	1635	1630
β -sheet	1639	1641	1643	1638
Random coil	1648	1650	1652	1646
α -helix	1657	1659	1661	1654
β -turns	1666	1668	1666	1662
β -turns	1682	1681	1683	1683
Aggregated strands	1691	1694	1695	1694



Spray-dried MPA



Freeze-dried MPA

