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Effects of extraction pH of chia protein isolates on functional properties

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24        **Abstract**

25            The aim of this work was to study the effect of the extraction pH on the functional  
26 properties of chia protein isolates (CPI). Samples were named as CPI10 or CPI12,  
27 according to their extraction pH, 10 or 12, respectively. Functional properties were  
28 significantly modified by the extraction pH. Color properties revealed that CPI12 presented  
29 a lower  $L^*$  ( $47.8 \pm 0.9$  for CPI10 and  $30 \pm 1$  for CPI12) due to enhanced protein-polyphenol  
30 interactions. Besides, a higher  $b^*$  value was obtained for CPI12 ( $7.0 \pm 0.3$  for CPI12 and  
31  $5.6 \pm 0.7$  for CPI10), as a result of a higher ash content. CPI12 showed a higher WAC  
32 probably due to a higher exposure of polar amino acids ( $4.4 \pm 0.1$  g/g and  $6.0 \pm 0.2$  g/g),  
33 whereas CPI10 showed a higher ability to bind oil ( $7.1 \pm 0.2$  g/g and  $6.1 \pm 0.2$  g/g for CPI10  
34 and CPI12, respectively). CPI10 proved more appropriate as an emulsion stabilizer than  
35 CPI12, which could be due to its higher surface hydrophobicity, protein solubility and  
36 negative net charge. The  $d_{4,3}$  ( $\mu\text{m}$ ) was  $29.5 \pm 0.4$  and  $20.4 \pm 0.3$  in emulsions stabilized with  
37 CPI12 and CPI10, respectively. Although both isolates underwent heat gelation, they  
38 exhibited a weak gel behavior. Overall, CPI10 may be more suitable for the food industry  
39 as a meat replacer or extender.

40    **KEYWORDS:** Vegetable protein; alkaline extraction; emulsion stability; heat  
41 gelation.

42

## 43 **Introduction**

44 The successful use of protein isolates or concentrates on food formulations depends  
45 mainly on the versatility of their functional properties, which are significantly influenced  
46 by the structural conformation of their proteins (Aluko & Yada, 1995; Salcedo-Chávez,  
47 Osuna-Castro, Guevara-Lara, Domínguez-Domínguez, & Paredes-López, 2002).

48 Kinsella and Melachouris (1976) have defined functional properties as those  
49 physicochemical properties that affect the behavior of proteins not only in food products  
50 but also during processing and storage. Properties such as formation and stability of  
51 emulsions, as well as viscosity and gelation are profoundly influenced by protein  
52 physicochemical properties such as conformation, hydrophobicity and thermal stability  
53 (Cordero-De-Los-Santos, Osuna-Castro, Borodanenko, & Paredes-López, 2005).

54 The most important factor which affects protein conformation is pH, since it modifies  
55 the charge and degree of unfolding of proteins (Valenzuela, Abugoch, Tapia, & Gamboa,  
56 2013). In particular, alkali and acid treatments during protein extraction through isoelectric  
57 precipitation are known to induce structural changes on proteins. Furthermore, there is  
58 sufficient evidence of a relationship between the extraction pH and the functional properties  
59 of vegetable protein isolates (Abugoch, Romero, Tapia, Silva, & Rivera, 2008; Aluko &  
60 Yada, 1995; Salcedo-Chávez et al., 2002; Valenzuela et al., 2013).

61 Chia, a Mesoamerican pseudocereal, is one of the non-conventional protein sources  
62 which has been increasingly studied in recent years (López, Galante, Robson, Boeris, &  
63 Spelzini, 2018). The composition of chia seeds results attractive as it is a good source of  
64 oil, protein and fiber, becoming thereby important for nutrition (Sandoval-Oliveros &

65 Paredes-López, 2012). The world claim for new protein sources along with the well-known  
66 health benefits of chia seeds has resulted in a remarkably increased demand of this  
67 pseudocereal in the functional food market.

68 Timilsena et al. (2016) have studied the physicochemical and functional properties of  
69 chia protein isolates obtained by extraction at pH 12 and dried by different methods. In  
70 addition, the structural properties of chia protein isolates (CPI) extracted at pH 10 or 12 and  
71 precipitated at pH 4.5 have been studied in a recent work (López et al., 2017). The protein  
72 content was similar in both isolates, being 782 g/kg and 775 g/kg for the extraction  
73 procedure at pH 10 or 12, respectively. Significant differences in the protein conformation  
74 were evinced. In particular, alkaline solubilization at pH 12 resulted in proteins with a  
75 higher content of unordered structure. However, how these conformational changes affect  
76 functional properties has not yet been evaluated.

77 The aim of this work was to study the effect of extraction pH (10 or 12) on some  
78 functional properties of chia protein isolates so as to explore and understand their behavior  
79 in food products.

## 80 **2. Materials and methods**

### 81 **2.1. Materials**

82 Commercial milled and partially defatted chia seeds were purchased at a local  
83 market from Sturla S.R.L. (Buenos Aires, Argentina). All the chemicals used were of  
84 analytical grade.

### 85 **2.2. Methods**

#### 86 **2.2.1. Protein isolation**

87 CPI were obtained by isoelectric precipitation, as described in a previous study  
88 (López et al., 2017). Briefly, milled and partially defatted chia seeds were mixed with  
89 distilled water (ratio 1:20), stirred for 30 min and then centrifuged at 10000 g for 15 min.  
90 The mucilaginous intermediate phase was removed, while chia proteins were recovered in  
91 the upper aqueous phase and in the bottom phase. The pH of the resulting slurry was  
92 adjusted to pH 10 or 12 with 1 mol/L NaOH and kept stirring for 1 h. After centrifugation  
93 at 10000 g for 15 min, the supernatant pH was adjusted to pH 4.5 with 1 mol/L HCl, while  
94 this slurry was kept stirring for 1 h to ensure the precipitation of chia proteins. The  
95 precipitate was recovered by centrifugation at 10000 g for 15 min. Proteins were  
96 redissolved in distilled water adjusted to pH 10 or 12, according to their extraction pH.  
97 Samples were finally freeze-dried and named as CPI10 or CPI12, depending on their  
98 extraction pH (10 or 12, respectively). Ash content was determined by the standard method  
99 AOAC (AOAC, 1990). Protein solubility was determined by dispersion of both isolates in  
100 distilled water at 10 g/L and the pH was adjusted to 7 with 1 mmol/L NaOH. Samples were  
101 stirred for 1 h and then centrifuged for 15 min at 10000 g. The concentration of soluble  
102 protein in the supernatant was determined by the bicinchoninic acid assay method (Smith et  
103 al., 1985). Protein solubility was expressed as a percentage of the total protein. Phenolic  
104 content was determined according to the methodology described by Arnous et al (Arnous,  
105 Makris, & Kefalas, 2002). The total polyphenol content was expressed as gallic acid  
106 equivalents.

### 107 **2.2.3. Color properties**

108 The color properties of the resulting isolates were evaluated by means of color  
109 digital analysis. Samples were photographed on a matte black background with a digital

110 camera (Canon EOS-Rebel T3) used in manual mode, as described by Soazo et al. (2015).  
111 The average values of the luminous and chromatic components were obtained using the  
112 graphic software Photoshop (Adobe Systems, Inc., San Jose, CA, USA), and were then  
113 converted into  $L^*$ ,  $a^*$  and  $b^*$ . The whiteness index (WI) was calculated according to  
114 Equation 1:

$$WI=L^*-3b^* \text{ (Equation 1)}$$

116

#### 117 **2.2.4. Water and oil absorption capacities**

118 The water and oil absorption capacities (WAC and OAC, respectively) of both CPI  
119 were determined according to Rodríguez-Ambriz et al. (2005). Briefly, 0.5 g of CPI10 and  
120 CPI12 were weighted and stirred into 5 mL deionized water or corn oil for 1 min. Then, the  
121 mixtures were kept at room temperature for 30 min and centrifuged at 1600 g for 25 min.  
122 The amount of supernatant was weighted so as to express the WAC or OAC as gram of  
123 water/oil absorbed per gram of protein isolate.

#### 124 **2.2.5. Surface hydrophobicity**

125 The surface hydrophobicity ( $S_0$ ) of aqueous dispersions of both CPI was determined  
126 according to Kato and Nakai (1980), using 1-anilino-8-naphtalene-sulfonate (ANS) as  
127 fluorescent probe. Samples were prepared in distilled water at a final concentration of 0.6  
128 g/L. Excitation and emission wavelengths were fixed at 380 and 484 nm, respectively. The  
129 fluorescence intensity of 4 mmol/L ANS was measured in the absence and presence of the  
130 different concentrations of each protein dispersion.  $S_0$  was determined as the initial slope of  
131 the plot of fluorescence intensity vs protein concentration.

## 132 2.2.6. Emulsion properties

### 133 2.2.6.1. Emulsion preparation

134 CPI were suspended at 30 g/L in buffer Tris-HCl 100 mmol/L pH 7 and stirred for 1  
135 h at 20 °C. Emulsions were prepared by homogenizing corn oil and protein suspension (oil  
136 volume fraction of 0.25) at 20000 rpm for 1 min with a high speed homogenizer device  
137 (Ultraturrax T-25, IKA Labortechnik, Karlsruhe, Germany) in order to study the effect of  
138 CPI on their stability.

### 139 2.2.6.2. Creaming stability

140 Immediately after preparation, emulsions were placed in 10 mL graduated tubes so  
141 as to study the creaming process. The creaming index (CI) was calculated after storage for  
142 24 h at 20 °C, according to Equation 2:

$$143 \quad CI (\%) = (H_s/H_t) * 100 \text{ (Equation 2)}$$

144 where  $H_s$  is the height of the serum phase and  $H_t$  is the total height of the emulsion (Shao  
145 & Tang, 2014).

### 146 2.2.6.3. Determination of particle size distribution

147 The particle size distributions were determined by laser diffraction using a Malvern  
148 Mastersizer 2000E analyzer (Malvern Instruments, Malvern, UK) associated with a wet  
149 dispersion unit (Hydro 2000MU, Malvern Instruments, Malvern, UK). The pump speed  
150 was fixed at 2000 rpm. Mean Sauter diameters ( $d_{3,2}$ ) and mean De Brouckere diameters  
151 ( $d_{4,3}$ ) were determined immediately after emulsion formation and after 24 h of storage at 20  
152 °C.



#### 153 **2.2.6.4. Rheological measurements**

154 The rheological properties of the emulsions stored for 24 h at 20 °C were evaluated  
155 using a controlled stress rheometer AR-G2 (TA Instruments, New Castle, USA), equipped  
156 with a 40 mm diameter stainless-steel plate geometry with a gap of 1 mm. For each  
157 measurement, 1 mL of each sample was carefully transferred to the rheometer. Temperature  
158 was fixed at 20 °C and controlled with a water bath (Julabo ACW100, Julabo Labortechnik,  
159 Seelbach, Germany) associated with the rheometer. The flow curves were obtained at an  
160 increasing shear rate from 0.1 to 100 1/s.

#### 161 **2.2.7. Heat-induced gelation of chia protein isolates**

162 To study heat-induced gelation of CPI10 and CPI12, suspensions were prepared  
163 from powder isolates at a concentration of 100 g/L in buffer Tris-HCl 100 mmol/L pH 10  
164 and stirred for 1 h at room temperature. Oscillatory rheological tests were carried out using  
165 a rheometer AR-G2 (TA Instruments, New Castle, USA), equipped with a stainless-steel  
166 plate geometry with a diameter of 40 mm and a gap of 1 mm. To prevent evaporation,  
167 samples were covered with a thin layer of low-density silicon oil. Samples were heated  
168 from 25 to 85 °C at a heating rate of 2 °C/min, kept at 85 °C for 10 min, and cooled to 25  
169 °C at a rate of 4 °C/min. Temperature was controlled with a water bath (Julabo ACW100,  
170 Julabo Labortechnik, Seelbach, Germany) associated with the rheometer. Strain and  
171 frequency were fixed at 1 % and 1 Hz, respectively. During the entire temperature ramp,  
172 the storage modulus ( $G'$ ) and loss modulus ( $G''$ ) were determined. Frequency sweep  
173 measurements were then carried out at 25 °C with a 1 % strain and a frequency range from  
174 0.1 to 10 Hz.

### 175 2.3. Statistical analysis

176 Experiments were run at least in triplicate. The effect of the extraction pH of chia  
177 proteins on the functional properties studied was determined by means of t-tests. Normality  
178 and equal variance assumptions were tested before performing parametric tests. Differences  
179 were considered significant when p-values (p) were lower than 0.05.

180

## 181 3. Results and Discussion

### 182 3.1. Color properties

183 Color properties of CPI10 and CPI12 were evaluated through digital analysis (Fig  
184 1).

185 Fig 1.

186 Some authors (Salcedo-Chávez et al., 2002; Steffolani et al., 2016) have stated that  
187 color in the protein isolates is the result of different compounds -including polyphenols-  
188 which bind to the proteins and co-precipitate during extraction, since oxidation of these  
189 slightly colored compounds results in highly colored products. Both isolates presented low  
190 L\* values, being lower for CPI12 ( $p < 0.0001$ ). As a result, a significantly lower WI value  
191 was obtained for CPI12 ( $p < 0.0001$ ). Although polyphenols are known to be present in chia  
192 seeds (Saphier, Silberstein, Kamer, Ben-Abu, & Tavor, 2017), their content in both isolates  
193 (0.133 mg gallic acid/mg CPI) showed no significant differences ( $p = 0.1489$ ), indicating  
194 that the protein-polyphenol interaction is not responsible for the different luminosity.

195 As regards  $a^*$  values, no significant difference was found between the two samples  
196 ( $p=0.2064$ ), while the  $b^*$  value was significantly higher for CPI12 ( $p=0.0278$ ).

197 The correlation among  $L^*$ ,  $b^*$  values and the ash content has already been reported  
198 for amaranth protein isolates (Shevkani, Singh, Rana, & Kaur, 2014). The ash content was  
199 higher for CPI12 than for CPI10 (near 80 and 40 g/kg for CPI12 and CPI10, respectively).  
200 Therefore, the lower  $L^*$  and higher  $b^*$  values obtained for CPI12 could account for its  
201 higher ash content.

202 Santillán-Álvarez et al. (2017) have also recently reported low  $L^*$  and WI values for  
203 chia protein flour ( $36 \pm 1$  and  $34 \pm 1$ , respectively). Our results show that the protein  
204 extraction procedure at pH 10 resulted in an increase in luminosity when compared to this  
205 flour.

### 206 **3.2. Protein solubility and absorption capacities**

207 Protein solubility was determined at pH 7 and resulted to be significantly lower in  
208 CPI12 ( $p<0.0001$ ) ( $68 \pm 1$  and  $90 \pm 3$  % soluble protein/crude protein, for CPI12 and  
209 CPI10, respectively). Protein denaturation during alkaline and acid treatments is known to  
210 induce protein aggregation. This effect has been previously reported for CPI12 (López et  
211 al., 2017), protein aggregation probably being responsible for the lower protein solubility.  
212 The higher protein solubility showed for chia isolates extracted at lower alkaline pH has  
213 also been previously reported for quinoa protein isolates (Abugoch et al., 2008; Ruiz, Xiao,  
214 van Boekel, Minor, & Stieger, 2016).

215 Water and oil absorption capacities of both isolates were determined and the results  
216 obtained are shown in Table 1.

217

Table 1.

218           The primary sites of water-protein interactions are the result of the presence of polar  
219 amino groups in the proteins, influencing the water-binding properties of a protein isolate,  
220 usually referred to as water absorption capacity. Differences in protein purity as well as in  
221 the conformational characteristics of a protein isolate results in different ability to bind  
222 water (Chavan, McKenzie & Shahidi, 2001).

223           CPI12 showed a higher WAC than CPI10 ( $p=0.0004$ ), probably due to the higher  
224 exposure of polar amino acids as a consequence of changes in protein conformation during  
225 extraction (Chavan et al., 2001; López et al., 2017). This high WAC was significantly  
226 higher than that previously reported by Olivos-Lugo et al. (2010). According to the  
227 methodology carried out, the hydration of the powder isolates enables the solubilization of  
228 the fraction of proteins soluble in distilled water. As this fraction is discarded by  
229 centrifugation, WAC may be attributed to the insoluble proteins. CPI12 showed lower  
230 solubility, therefore, the higher insoluble fraction of proteins could be responsible for the  
231 higher WAC.

232           Despite this difference, both samples proved to have higher WAC than those  
233 reported for other pseudocereal protein isolates (Abugoch et al., 2008; Steffolani et al.,  
234 2016). This fact supports the idea of the future use of these food additives in formulations,  
235 so as to diminish water loss in cooked products as well as to improve yields.

236           As regards their capacity to bind oil, both isolates showed high OAC values, being  
237 higher for CPI10 ( $p=0.0051$ ). This result is important since the ability of proteins to bind oil

238 is connected with the enhancement of flavor retention and the improvement of mouth feel  
239 (Kinsella & Melachouris, 1976).

240 The fact that both isolates showed high binding capacities is interesting since it  
241 shows a good balance between polar aminoacids and hydrophobic residues in both resulting  
242 protein isolates.

### 243 **3.3. Surface hydrophobicity**

244 Surface hydrophobicity ( $S_0$ ) is particularly related to the extent of hydrophobic  
245 aminoacids which are exposed on the protein surface. Therefore, it influences protein-lipid  
246 as well as protein-protein interactions (Timilsena et al., 2016).  $S_0$  of both isolates were  
247 determined and proved to be higher for CPI10 ( $p=0.0072$ ) (Table 1). This fact is related to  
248 the higher OAC obtained for CPI10, which shows higher exposure of hydrophobic  
249 aminoacids, resulting in an enhanced protein-lipid interaction.

250 It has already been reported that the extraction of chia proteins at pH 12 leads to a  
251 more unordered conformation, with a higher content of random structure (López et al.,  
252 2017). In this conformation, hydrophobic aminoacids are expected to be surface-exposed to  
253 a higher extent. However, the lower  $S_0$  obtained suggests the formation of protein-protein  
254 aggregates through hydrophobic patches. If these aggregates remain in the solution, the  
255 fluorescent probe ANS has fewer hydrophobic patches available to interact, since the  
256 protein-protein interaction is favored. On the other hand, if these aggregates are insoluble,  
257 protein-protein aggregates may precipitate during the assay. Both effects may explain the  
258 lower  $S_0$  obtained, as well as the lower protein solubility for CPI12.

259 Besides, the formation of protein aggregates in CPI12 is consistent with the results  
260 previously obtained through laser diffraction, since a protein suspension of CPI12 revealed  
261 a higher mean diameter than CPI10 (López et al., 2017).

### 262 **3.4. Emulsion properties**

#### 263 **3.4.1. Creaming stability**

264 The creaming index (CI) of emulsions stabilized with CPI10 and CPI12 was  
265 measured after 24 h of emulsion preparation, as described in Section 2.2.6.2. Results are  
266 shown in Table 2. A significantly higher CI was observed in emulsions stabilized with  
267 CPI12 ( $p=0.0009$ ).

268 The differences obtained may be explained by the fact that chia proteins extracted at  
269 different alkaline pHs show different abilities to generate repulsive interactions among oil  
270 droplets. Surface hydrophobicity, electrical charges and surface activity are important  
271 factors that modify colloidal interactions among oil droplets (Onsaard, Vittayanont, Srigam,  
272 & McClements, 2006). In particular, high solubility is known to be necessary for rapid  
273 migration to the oil-water interface (Karaca, Low, & Nickerson, 2011). Protein solubility at  
274 pH 7 was higher for CPI10 than for CPI12. Therefore, a higher soluble protein  
275 concentration may increase the coverage of oil droplets, favoring the stabilization of the  
276 colloid (Zayas & Lin, 1989). Besides, in order to display surface activity, proteins should  
277 exhibit hydrophobic patches as well as a large net charge to prevent droplet aggregation  
278 (Karaca et al., 2011). Proteins present in CPI10 showed a higher zeta potential value than  
279 those present in CPI12 at neutral pH, which is related to a higher negative charge.

280 Moreover, the higher  $S_0$  exhibited in the CPI10 dispersion could also contribute to a higher  
281 stabilized oil-water interface, thus resulting in less creaming.

### 282 3.4.2. Determination of particle size distribution

283 Fig 2. shows the particle size distribution of emulsions stabilized with CPI10 and  
284 CPI12. Since similar distributions were obtained in the different replicates, only one data  
285 set is shown for each sample.

286 Fig. 2

287 Both emulsions were polydisperse samples, with a multimodal particle size  
288 distribution. However, both samples showed one predominant peak. The magnitude and  
289 location of that peak are different in both samples, being slightly higher and shifted to a  
290 lower particle size in emulsions stabilized with CPI10. In both samples, a minor population  
291 of particles whose sizes are significantly lower can be distinguished. The distribution of  
292 emulsions stabilized with CPI12 presented a shoulder corresponding to a population with  
293 higher particle sizes. Thus, the particle size distribution for emulsions stabilized by CPI12  
294 was broader than for those stabilized by CPI10.

295 The analysis of mean diameters showed significant differences between both  
296 samples. Results are shown in Table 2.

297 Table 2.

298 Emulsions stabilized with CPI12 showed larger particle sizes than emulsions  
299 stabilized with CPI10, in accordance with the higher CI value obtained. The higher  $d_{3,2}$   
300 value obtained in emulsions stabilized with CPI12 ( $p < 0.0001$ ) is related to the lower  
301 specific surface area, whereas their higher  $d_{4,3}$  values ( $p < 0.0001$ ) suggest either the

302 formation of large droplets due to low interfacial activity or the assembly of individual  
303 droplets into larger flocs (Intarasirisawat, Benjakul, & Visessanguan, 2014).

304 Coalescence and creaming are instability processes governed by the average droplet  
305 size. Stoke's law explains the effect of the aqueous phase viscosity ( $\eta_2$ ), particle radius ( $r$ ),  
306 acceleration due to gravity ( $g$ ) and oil and aqueous phase densities ( $\rho_1$  and  $\rho_2$ , respectively)  
307 on the velocity of creaming of oil droplets ( $v$ ) by using Equation 3:

$$308 \quad v = \frac{2gr^2(\rho_1 - \rho_2)}{9\eta_2} \text{ (Equation 3)}$$

309 As a consequence, emulsions with a reduced droplet size, such as those obtained  
310 through stabilization with CPI10, showed better stability in phase separation processes  
311 which are mediated by gravitation (Thaiphanit, Schleining, & Anprung, 2016).

312 Emulsion incubation at 20 °C for 24 h did not change the droplet size distribution in  
313 emulsions stabilized with CPI10, showing no significant differences in  $d_{4,3}$  diameter  
314 ( $p=0.1671$ ). However, emulsions stabilized with CPI12 showed higher  $d_{4,3}$  values after  
315 storage ( $p=0.0019$ ), which increased from  $29.5 \pm 0.4 \mu\text{m}$  to  $32 \pm 1 \mu\text{m}$ , suggesting further  
316 droplet flocculation or coalescence.

### 317 **3.4.3. Rheological measurements**

318 The flow behavior of both emulsions stored for 24 h at 20 °C was determined  
319 according to Section 2.2.6.4.

320 Non-ideal behavior was exhibited in both cases, with a reduction in the apparent  
321 viscosity as the shear rate increased. This flow behavior is referred to as shear thinning or  
322 pseudoplastic and it is known to be the most common type of non-ideal flow behavior in



323 food emulsions. Systems that are usually weakly flocculated are usually shear-thinning.  
324 This behavior is a consequence of weak associative interactions among the particles, which  
325 gives raise to the formation of a weak elastic gel-like network (Torres, Iturbe, Snowden,  
326 Chowdhry, & Leharne, 2007). Flocs may become deformed and disrupted as the shear rate  
327 increased, becoming elongated and aligned with the shear flow, offering less resistance to  
328 flow and hence reducing the apparent viscosity (McClements, 2015).

329 Over the shear rates studied, the flow behavior of both emulsions was properly  
330 described by a power law equation (Equation 4):

$$331 \quad \tau = K\dot{\gamma}^n \text{ (Equation 4)}$$

332 where  $\tau$  is defined as the shear stress,  $\dot{\gamma}$  as the shear rate,  $K$  is the flow consistency  
333 and  $n$  is flow behavior index.  $K$  and  $n$  parameters were determined for each sample and are  
334 shown in Table 2. No significant differences between both samples were obtained ( $p > 0.05$ ).  
335 Therefore, the alkaline extraction of chia proteins at the different pHs studied did not  
336 change the flow behavior of the emulsions stored for 24 h.

337 Apparent viscosity recorded at a shear rate of  $100 \text{ s}^{-1}$  (Table 2) did not show  
338 significant differences between both isolates ( $p > 0.05$ ).

339 To summarize, both emulsions showed differences in the creaming index as well as  
340 in the particle size distribution but no differences in the flow behavior after storage were  
341 observed. The higher emulsifying activity of CPI10 enabled the formation of particles with  
342 a lower mean size after storage due to a higher protein solubility and surface  
343 hydrophobicity. It is already known that when flocs are formed, they immobilize an amount  
344 of continuous phase within themselves (Pal & Rhodes, 1989). The smaller flocs formed by

345 CPI10 are believed to form an open packing, entrapping a larger amount of aqueous phase;  
346 whereas the larger flocs formed by CPI12 induced the formation of a close packing with a  
347 smaller amount of continuous phase (McClements, 2015). It may be proposed that  
348 emulsions stabilized by CPI10 formed a more hydrated cream phase than that formed by  
349 emulsions stabilized with CPI12, stabilizing them against coalescence (Palazolo,  
350 Sorgentini, & Wagner, 2004).

### 351 **3.5. Heat-induced gelation of chia protein isolates**

352 The heat-induced gelation behavior of CPI10 and CPI12 was studied through  
353 oscillatory rheological tests. Changes in  $G'$  (storage modulus) and  $G''$  (loss modulus) were  
354 analyzed during the entire gelation process. As the rheograms obtained from the different  
355 replicates did not show significant differences, only one data set for each sample is shown  
356 in Fig. 3.

357 Fig. 3.

358 During heating, both moduli increased, keeping  $G'$  lower than  $G''$ , until a certain  
359 temperature at which  $G'$  overtook  $G''$ . This temperature, referred to as gel temperature  
360 ( $T_{gel}$ ) was  $80 \pm 3$  °C for CPI10 and  $62 \pm 5$  °C for CPI12, respectively. In a previous study, a  
361 higher degree of protein denaturation for CPI12 was reported (López et al., 2017). Thus, the  
362 greater tendency of these proteins to aggregate may lead to a lower  $T_{gel}$ . Besides, the higher  
363 content of ordered structure shown in CPI10 is might be responsible for the higher  $T_{gel}$   
364 observed.

365 Recently, a  $T_{gel}$  of about 70 °C has been reported for amaranth and pea protein  
366 isolates (Ruiz et al., 2016). Quinoa protein isolates showed  $T_{gel}$  which varied from 64.6 to  
367 87.36 °C (Kaspchak et al., 2017).

368 However, both  $T_{gel}$  were significantly lower than the denaturation temperature  
369 previously reported from DSC studies for aqueous dispersions of chia protein fractions or  
370 isolates (López et al., 2018). This may be attributed to the dissolution of both isolates at pH  
371 10, a procedure which ensured high protein solubility. A lower denaturation temperature in  
372 pea protein has been reported as an effect of the protein dispersion at alkali pH (Meng &  
373 Ma, 2001). Moreover, this effect may be caused by the partial unfolding of proteins, which  
374 results in the exposure of functional groups that interact with each other, as recently  
375 described for quinoa protein gels (Kaspchak et al., 2017).

376 At crossover,  $G'$  was higher for CPI12 ( $p=0.0440$ ), which is consistent with a higher  
377 gel strength. Besides, the increase in the storage modulus during the heating ramp was  
378 significantly higher for CPI12 ( $p<0.0001$ ), significantly enhancing the formation of the gel  
379 network structure, possibly due to an increased formation of disulfide bonds.

380  $G'$  kept increasing until the heating ramp finished, and became even higher during  
381 the cooling stage. Such increase, referred to as gel reinforcement ( $G_r$ ), which indicates the  
382 gel network strengthening, was calculated as the difference between  $G'$  at 85 and 25 °C. At  
383 this stage, crosslinking continued and there was a slower formation and rearrangement of  
384 the gel structure. A higher  $G_r$  was obtained in gels formed from CPI12 ( $p<0.05$ ), suggesting  
385 that attractive forces such as van der Waals and hydrogen bonding were enhanced in the  
386 aforementioned sample.

387 According to Avanza et al. (2005), the  $\tan \delta$  values obtained after the heating and  
388 cooling ramps showed that both systems exhibit weak gel behavior. However, the type of  
389 gel formed is more adequately described by the dependence on frequency of  $G'$  and  $G''$ . In  
390 this context, frequency sweep measurements for CPI10 and CPI12 are shown in Fig. 4.

391 Fig. 4

392 In contrast with covalent/chemical gels, physical gels show frequency dependence.  
393 This effect was observed in both samples. Besides, CPI10 showed  $G'$  and  $G''$  crossover,  
394 which is consistent with a gel formed by an entanglement of molecules, called  
395 “entanglement network systems” (Spotti, Tarhan, Schaffter, Corvalan, & Campanella,  
396 2017).

397 It is worth highlighting that both isolates could undergo heat gelation under the  
398 conditions studied. Ruiz et al. (2016) have reported that quinoa proteins extracted at high  
399 pH (10 and 11) could not undergo heat gelation due to a high degree of protein  
400 denaturation. Therefore, chia proteins were more suitable to support extreme extraction  
401 conditions than quinoa proteins.

#### 402 **4. Conclusions**

403 The present study revealed that extraction pH (10 or 12) significantly influences the  
404 functional properties of CPI. Color properties showed an enhanced protein-polyphenol  
405 interaction in CPI12, which resulted in lower  $L^*$  and WI values. Besides, the higher  $b^*$   
406 obtained is probably due to its higher ash content. The study of binding properties proved  
407 that both isolates present well-balanced polar and hydrophobic aminoacids in their protein  
408 composition. As regards their ability to stabilize emulsions, CPI10 was more adequate. The

409 droplet size was lower in this sample, which is explained by its higher surface  
410 hydrophobicity, net negative charge and higher protein solubility. As a consequence, the  
411 creaming process is lesser than in emulsions stabilized with CPI12, which showed higher  
412 droplet size. CPI10 may be a suitable additive in meat products or emulsion-type sausages,  
413 as a meat replacer or extender, not only because of its ability for fat emulsification but also  
414 because of its high WAC and OAC, which could improve cooking yields, enhance flavor  
415 retention and improve mouthfeel. Heat-induced gelation of both CPI resulted in weak gels,  
416 CPI10 gel being formed by an entanglement of molecules. Even though both samples  
417 underwent gelation due to heat treatment, they were not able to develop a strong gel  
418 network. Functional properties studied in the present work might be closely related to the  
419 physicochemical properties previously published for both isolates.

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541

542 **Figure captions**

Fig 1. Color digital analysis of chia protein isolates obtained at pH 10 (CPI10) or 12 (CPI12). The inset shows a photograph of each isolate.

Fig. 2. Particle size distribution of freshly made emulsions (oil volume fraction of 0.25) stabilized with chia proteins extracted at pH 10 (---) or 12 (—). For emulsion formation, isolates were dispersed at 30 g/L in buffer Tris-HCl 100 mmol/L pH 7.

Fig. 3. Storage ( $G'$ ;  $\bullet$ ) and loss ( $G''$ ;  $\circ$ ) moduli as a function of the temperature ramp (—) for the heat gelation process of chia proteins extracted at pH 10 (A) or 12 (B). Strain and frequency were fixed at 1 % and 1 Hz, respectively. Samples were prepared from powder isolates at a concentration of 100 g/L in buffer Tris-HCl 100 mmol/L pH 10.

Fig. 4. Frequency sweep measurements of heat-induced gels of chia protein isolates obtained at pH 10 ( $\Delta$ ;  $\blacktriangledown$ ) or 12 ( $\bullet$ ;  $\circ$ ). The filled symbols represent the elastic modulus ( $G'$ ) while open symbols correspond to the loss modulus ( $G''$ ). Strain and temperature were fixed at 1% and 25 °C, respectively.

Table 1. Binding properties and surface hydrophobicity of chia proteins extracted at pH 10 (CPI10) or 12 (CPI12).

	<b>CPI10</b>	<b>CPI12</b>
<b>WAC (g water absorbed/g protein isolate)</b>	4.4 ± 0.1 <sup>a</sup>	6.0 ± 0.2 <sup>b</sup>
<b>OAC (g oil absorbed/g protein isolate)</b>	7.1 ± 0.2 <sup>a</sup>	6.1 ± 0.2 <sup>b</sup>
<b>Surface hydrophobicity, S<sub>0</sub></b>	23.2 ± 0.7 <sup>a</sup>	13 ± 3 <sup>b</sup>

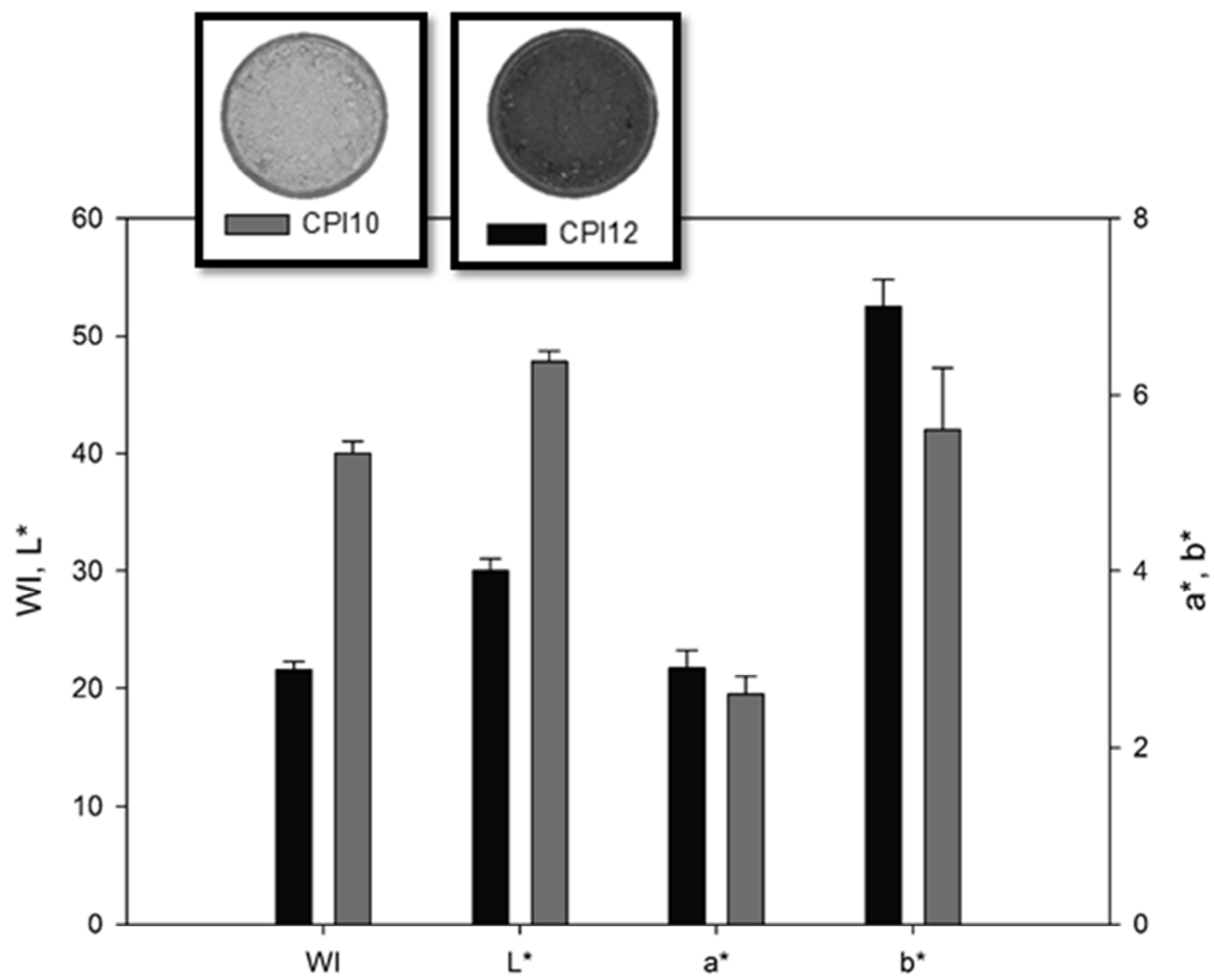
WAC= water absorption capacity; OAC= oil absorption capacity

Means ± standard deviation of triplicate assays. Mean values in the same row followed by the same letter are not significantly different (p > 0.05)

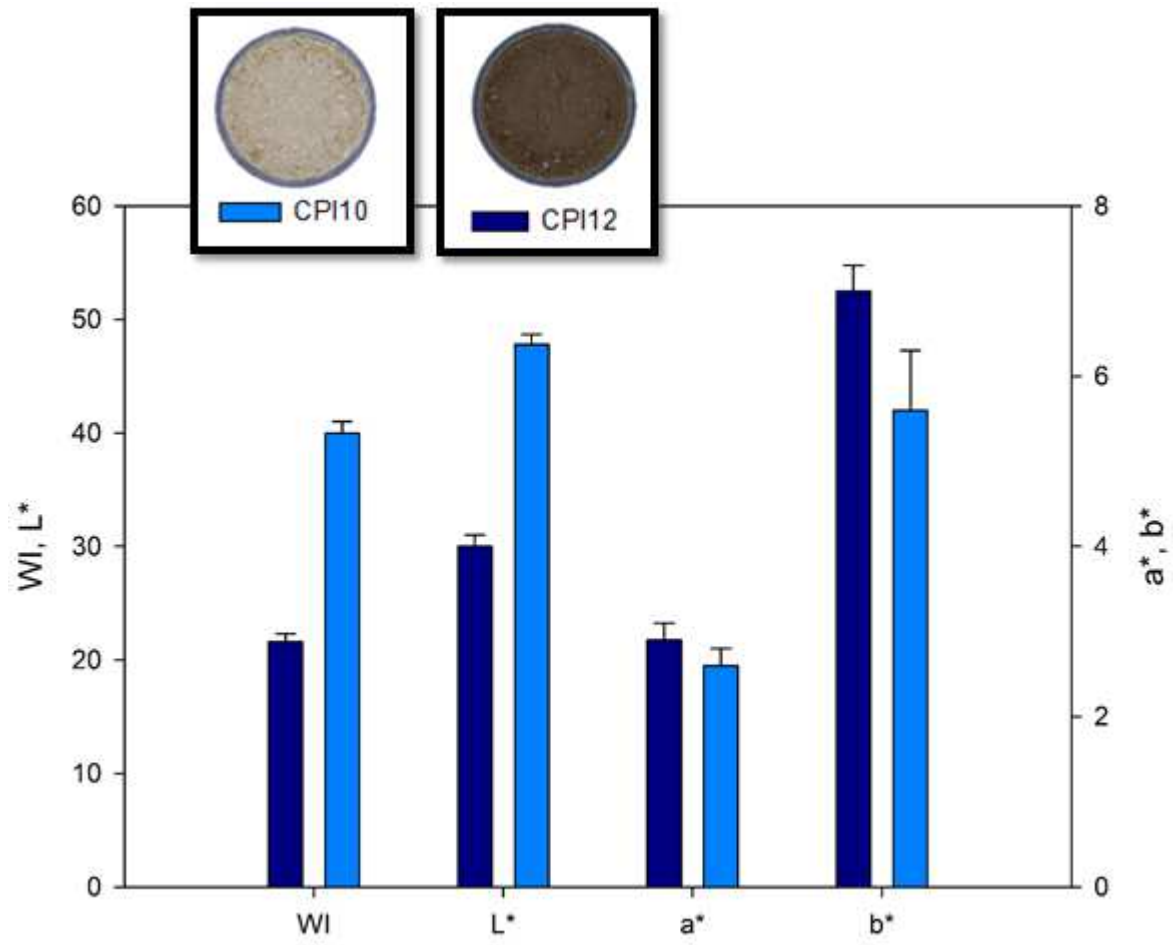
Table 2. Properties of emulsions stabilized with chia protein isolates obtained at pH 10 (CPI10) or 12 (CPI12) after 24 h of storage at 20 °C.

	<b>CPI10</b>	<b>CPI12</b>
<b>CI (%)</b>	1.5 ± 0.7 <sup>a</sup>	4.5 ± 0.7 <sup>b</sup>
<b>De Brouckere diameters, d<sub>4,3</sub> (µm)</b>	20.4 ± 0.3 <sup>a</sup>	29.5 ± 0.4 <sup>b</sup>
<b>Sauter diameters, d<sub>3,2</sub> (µm)</b>	11.4 ± 0.1 <sup>a</sup>	14.44 ± 0.04 <sup>b</sup>
<b>Flow consistency, K</b>	0.05 ± 0.01 <sup>a</sup>	0.05 ± 0.02 <sup>a</sup>
<b>Flow behavior index, n</b>	0.87 ± 0.01 <sup>a</sup>	0.8 ± 0.2 <sup>a</sup>
<b>Apparent viscosity (Pa*s)</b>	0.030 ± 0.002 <sup>a</sup>	0.018 ± 0.003 <sup>a</sup>

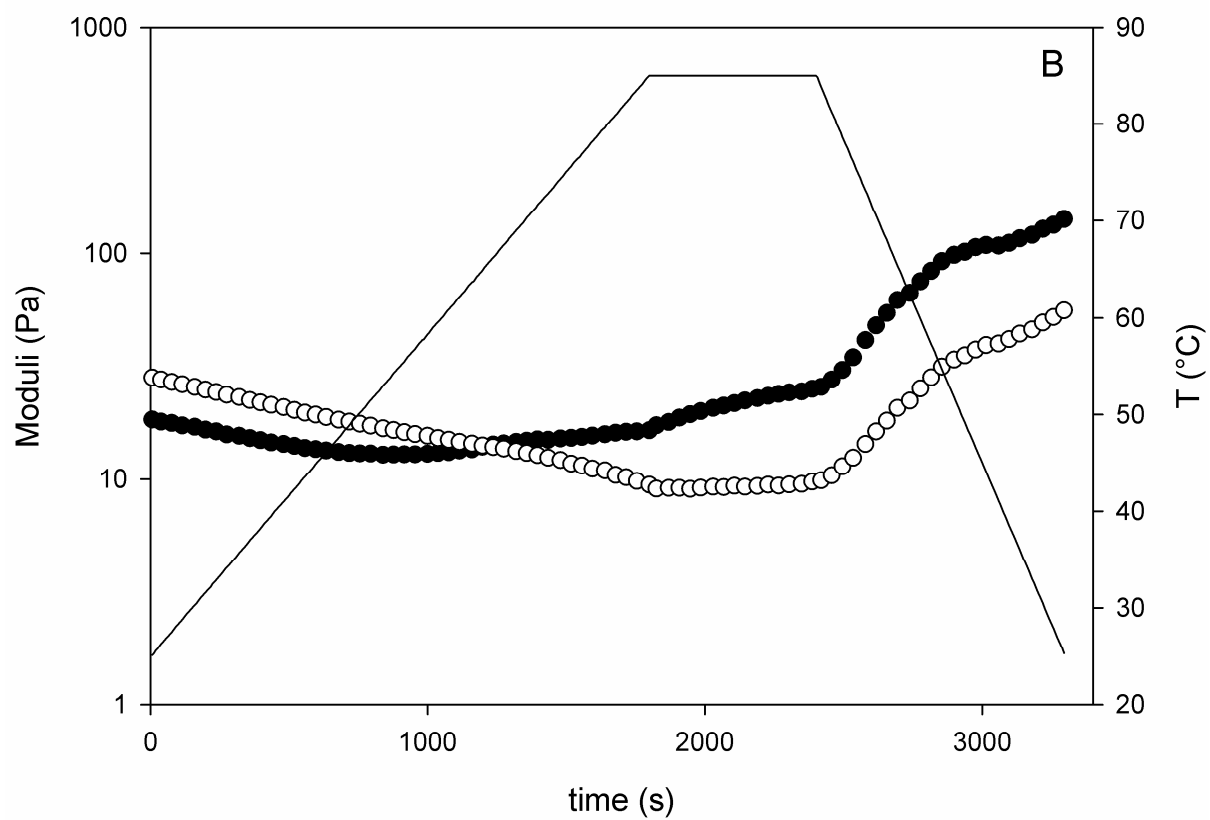
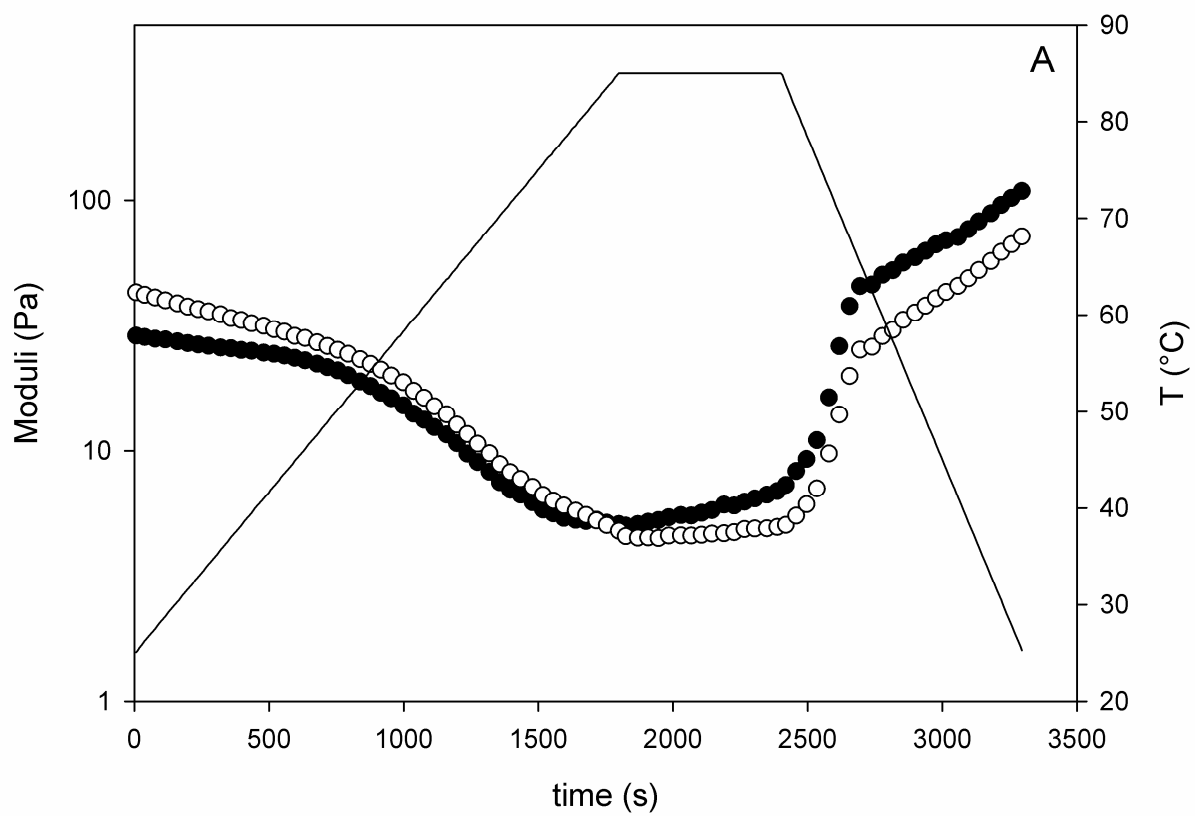
Means ± standard deviation of triplicate assays. Mean values in the same row followed by the same letter are not significantly different (p > 0.05).

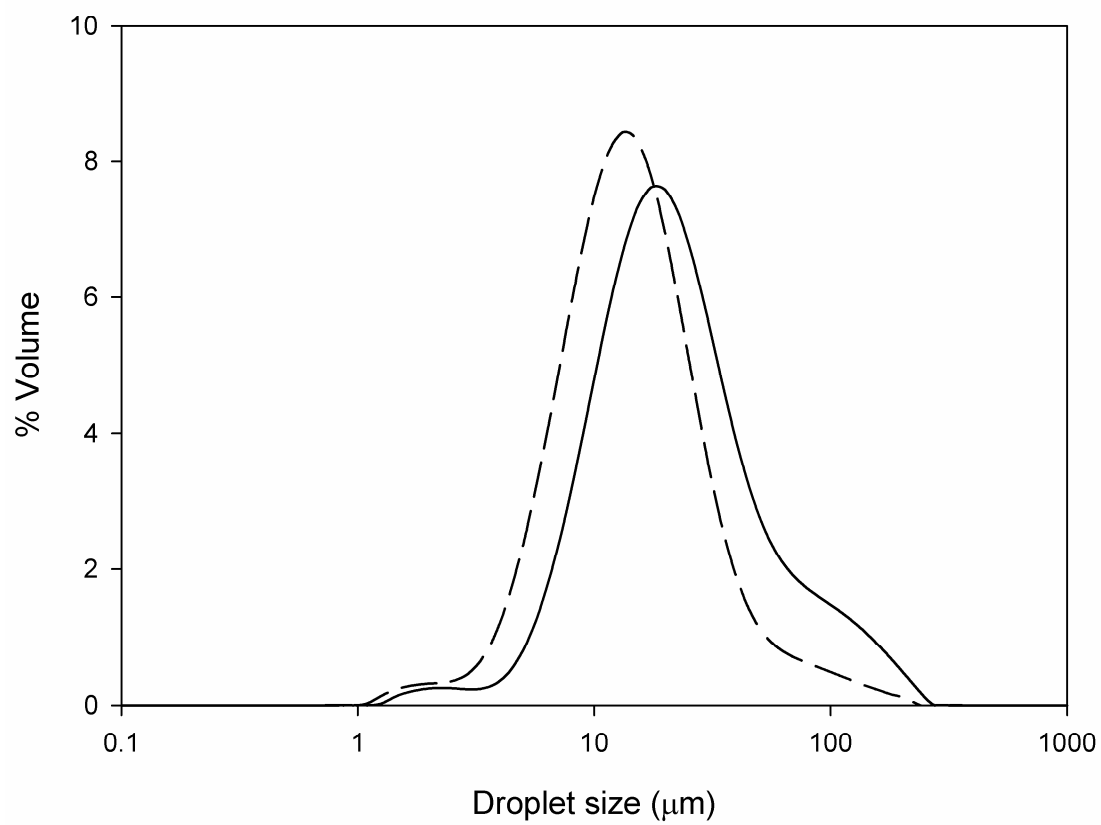


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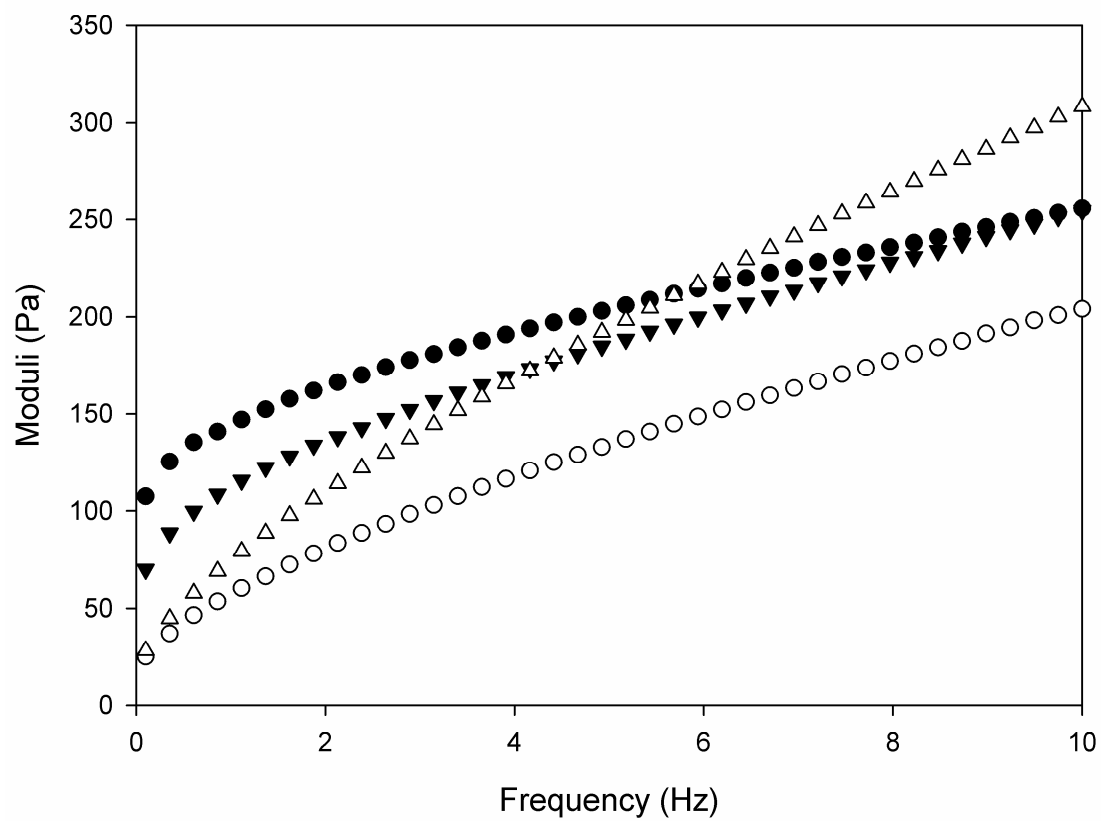


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**Highlights**

Protein isolates were obtained from chia seeds by extraction at pH 10 or 12

Chia protein isolates obtained at pH 10 showed higher emulsion stability

Weak gels were obtained after heat gelation of both isolates

The extraction pH influenced the functional properties of chia protein isolates