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# Combined effects of ionic strength and enzymatic pre-treatment in thermal gelation of peanut proteins extracts.

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## Abstract:

Peanut proteins are mostly composed of arachins and conarachins, globular proteins that can form gels under thermal denaturation or enzymatic treatment. We explore here how ionic strength (0.5 M or 0.8 M) and gelation process (a thermal treatment preceded or not by an enzymatic pre-treatment) affect peanut protein gel properties. Gel formation and final properties are characterized by rheology, and gel structure by confocal microscopy. We show that the ionic strength imposed during protein extraction determines the arachins/conarachins ratio, and that conarachins-rich samples give stronger gels, which is attributed to their higher content in free thiol groups and lysine residues. The gel storage modulus exhibited a power-law dependence with the protein concentration, which exponent depends on the gelation process. Rheological results, together with confocal microscopy imaging, showed that an enzymatic pre-treatment results in denser structures.

## Keywords:

Plant proteins, hydrogels, heat-set, transglutaminase, fractal dimension, structure, rheology, elastic modulus, confocal microscopy

## 34 1. Introduction

1 35 Peanut is a worldwide grown legume of high nutritional interest, characterized by a high energy  
2 36 density, about 550 kcal/100 g (Arya, Salve, & Chauhan, 2016). The major components of peanut  
3 37 are lipids (~50wt%), mostly composed of mono-unsaturated fatty acids (Brown, Cater, Mattil, &  
4 38 Darroch, 1975; Ciquel, 2017) associated with high HDL cholesterol level and improved heart  
5 39 condition (Feldman, 1999). Peanut is also rich in proteins (~25wt%), characterized by an elevated  
6 40 protein amino acid score (PDCAAS), between 0.5 and 0.7 (Suárez López, Kizlansky, & López,  
7 41 2006). This value is nutritionally equivalent to the one of animal proteins for human health and  
8 42 proper growth (Arya et al., 2016; Toomer, 2017). Peanut proteins also present functional  
9 43 properties for emulsification, foam formation or gelation (Monteiro and Prakash 1994; Yu et al.  
10 44 2007).

11 45 About 90wt% of peanut proteins are globular proteins, classified into three main families: the  
12 46 arachins, the conarachins I, and the conarachins II (Breese, 1917; Monteiro & Prakash, 1994).  
13 47 Arachins make up for about 66wt% of the peanut proteins. SDS-PAGE analysis shows the  
14 48 presence of 4 arachins subunits: 3 acidic subunits of 35 kDa, 37 kDa and 42 kDa and 1 basic  
15 49 subunit of 22 kDa (Hu, Zhao, Sun, Zhao, & Ren, 2011). Conarachins altogether make up for about  
16 50 30wt% of the peanut proteins. Conarachins I (13wt% of the peanut proteins) are contained in the  
17 51 cytosol while conarachins II (16wt% of the peanut proteins) are contained in protein bodies (the  
18 52 aleurones, that also contain arachins). Their composition differ: conarachin I is a trimer  
19 53 composed of small molecular weight polypeptides (18 kDa, 17 kDa, 15,5 kDa), while conarachin  
20 54 II is a trimer of three 64 kDa units (Toomer, 2017).

21 55 In solution, globular proteins behave in their native configuration as colloidal objects stabilized  
22 56 by repulsive interactions (Boire et al., 2019). During food processing, thermal treatment  
23 57 enzymatic action or acidification, may result in protein gelation, giving rise to solid-like behaviors  
24 58 associated with appreciated textural properties (Totosaus, Montejano, Salazar, & Guerrero,  
25 59 2002).

26 60 Thermal treatment requires a temperature higher than the protein denaturation temperature,  
27 61 so that proteins unfold and expose their buried hydrophobic amino acid residues. They  
28 62 subsequently aggregate into a percolating network through covalent and non-covalent  
29 63 interactions. For peanut flour suspensions, a thermal treatment above 90 °C results in gelation,  
30 64 the gels being harder as the flour (and protein) content increases (Davis, Gharst, & Sanders, 2007;

65 Kain & Chen, 2010). Increasing the ionic strength from 0 M to 0.5 M results in “weaker” gels  
66 (Schmidt, Illingworth, & Ahmed, 1978). A pre-treatment of the suspension with transglutaminase  
67 delays the gelation during thermal treatment, and results in softer gels (Gharst, Clare, Davis, &  
68 Sanders, 2007). A similar behavior is also observed in peanut protein isolate suspensions (Chen,  
69 Huang, Wang, Li, & Adhikari, 2016; Jiao, Shi, Liu, et al., 2018).

70 During enzymatic gelation, protein-protein covalent bonds are formed. For example, the  
71 microbial transglutaminase (mTG) forms  $\epsilon$ -( $\gamma$ - glutamyl) lysine intra- and inter-molecular bonds  
72 (Heck, Faccio, Richter, & Thöny-Meyer, 2013). This procedure was successfully applied on peanut  
73 protein isolates (Jiao, Shi, Wang, & Binks, 2018). Besides both arachins and conarachins are  
74 involved in the gel network, conarachins are more effectively cross-linked by mTG (Feng et al.,  
75 2014; Sun, Xiong, Bu, & Liu, 2012).

76 Acidification also induces gelation through the reduction of repulsive interactions between  
77 proteins as the protein net charge decreases. The storage modulus of peanut protein isolate gels  
78 formed by acidification follows a power-law behavior as a function of the concentration, that is  
79 reminiscent of fractal structures observed on colloidal gels (Bi et al., 2019).

80 The relatively low cost of peanuts, their interesting nutritional properties and the gelling capacity  
81 of their proteins paves the way for the production of new food products. Recent works reported  
82 on the formation of solid-like structures by processing grinded peanuts suspensions with  
83 transglutaminase (Basse, D’Huart, Lorand, Dupas, & Saiter, 2018; Patent No. WO 2019/073174  
84 Al, 2018), or by combining a transglutaminase treatment with an heat/high pressure treatment  
85 (115 °C, 0.17 MPa) to form tofu-like gels (Guo, Hu, Wang, & Liu, 2018). Textural properties can  
86 be tuned by the nature and amount of proteins solubilized in the aqueous phase, that is ionic-  
87 strength dependent, and by the gelation process parameters, but this requires a better  
88 understanding of processing-structure relationship.

89 We investigate here the gelation of protein extracts representative of the protein fraction  
90 solubilized when a peanut suspension is grinded in salted (NaCl) water. These extracts are  
91 obtained at two ionic strengths, 0.5 M and 0.8 M, to produce suspensions with different  
92 conarachins/arachins ratio. We first investigate by rheology the gelation of such suspensions  
93 through a thermal treatment at 95 °C, preceded or not by an enzymatic pre-treatment, at the  
94 two ionic strengths. Then, gel structure is characterized by confocal microscopy.

95

## 96 2. Materials & Methods

1 97

### 4 98 **2.1 Materials**

6 99 Dehulled peanuts from a blanched Runner High Oleic variety are bought by bags of 25 kg  
7  
8 100 (Bredabest BV, Netherlands), and stored in plastic containers of 25 kg under vacuum, at 4 °C.

10 101 Microbial transglutaminase (EC 2.3.2.13, Activa® EB, activity = 34 U/g) from *Streptovercillium*  
11  
12 102 *mobarraense* is supplied by Ajinomoto (Japan).

15 103

### 18 104 **2.2 Methods**

#### 20 105 **2.2.1 Sample preparation**

##### 22 106 2.2.1.1 Protein extraction and freeze-drying

24 107 Blanched white peanuts are first coarsely grinded for 2-3 min (MC15 grinder from Stephan,  
25  
26 108 Belgium) by batches of about 5 kg. The resulting paste is stored at 4 °C in hermetically sealed  
27  
28 109 containers and used within 4 months. For protein extraction, 295 g of this paste are mixed with  
29  
30 110 685 g of tap water and 20 or 32 g of sodium chloride (La Baleine Fine Sea Salt, France) to reach  
31  
32 111 an ionic strength (IS) of 0.5 M or 0.8 M, respectively. This suspension is then mixed and finely  
33  
34 112 grinded at room temperature for 15 min at 1100 rpm in a Thermomix TM5 (Vorwerk, Germany),  
35  
36 113 to reach an average particle size of about 25µm.

38  
39 114 The suspension is then dispatched in 50 mL centrifuge tubes, and centrifuged at 10 000 g for 2 h  
40  
41 115 (Sigma 3-18K centrifuge from Fisher Scientific, USA). Three fractions were obtained: the  
42  
43 116 supernatant, which contains most of the lipids, the pellet, which contains the insoluble particles,  
44  
45 117 and the intermediate aqueous phase, which contains in particular the solubilized proteins. This  
46  
47 118 intermediate fraction is turbid, suggesting the presence of proteins aggregates or impurities that  
48  
49 119 are not eliminated by centrifugation, however, we choose not to purify further these extracts in  
50  
51 120 order to keep all the solubilized/suspended proteins in the aqueous intermediate phase. The  
52  
53 121 corresponding protein extraction yields, determined with the Kjeldhal method (see below)  
54  
55 122 ranged from 41%±7% w/w (for IS = 0.5 M) to 54%±13% w/w (for IS = 0.8 M) of the total amount  
56  
57 123 of proteins present in the mix. This fraction is collected, frozen at -80 °C, and freeze-dried until  
58  
59 124 complete extraction of the water (Alpha 2-4 LD<sub>plus</sub> freeze-dryer from Martin Christ, Germany).  
60  
61 125 Freeze-dried protein powders are stored at 4 °C in sealed containers and used within 2 weeks.

## 126 2.2.1.2 Protein suspensions preparation

1 127 Protein suspensions from 4wt% to 16wt% are prepared by batches of 60 mL from the freeze-  
2 128 dried powder dispersed in distilled water under magnetic stirring for 15 min. To ensure constant  
3 129 ionic strength, suspensions are dialyzed in dialysis bags (Spectra/Por, molecular weight cut-off  
4 130 3 kDa from Spectrum, USA) against a 0.5 M or 0.8 M NaCl solution (renewed three times) for  
5 131 48 h. Protein suspensions (pH=6.2±0.2) are then collected and weighed to determine the dilution  
6 132 factor and assess their final protein concentration.

## 13 133 2.2.2 Protein characterization

### 14 134 2.2.2.1 Protein content

15 135 Nitrogen contents in the freeze-dried powders are determined in triplicate on 0.1g of sample,  
16 136 with the Kjeldhal method (Speed Digester K-439 from BUCHI, France), using a protein-nitrogen  
17 137 coefficient of 5.46 (Misra, 2001). The mineralization gradient is 300 °C for 20 min, 400 °C for 20  
18 138 min, and 520 °C for 90min.

### 25 139 2.2.2.2 Total free sulphhydryl content

26 140 Total free sulphhydryl content (TFS) is determined in triplicate using Ellman's reagent. Ellman's  
27 141 reagent (5,5-dithiobis-2-nitrobenzoic acid, DTBN) at a concentration of 4 mg/mL is prepared in  
28 142 an urea Tris-glycine EDTA buffer (8 M urea, 0.086 M Tris, 0.09 M glycine, 4 mM EDTA; pH 8.0).  
29 143 500 µL of the protein suspension with a protein concentration  $C_p \approx 5-10$  mg/mL are mixed with  
30 144 2.5 mL of buffer and 20 µL of Ellman's reagent. Samples are gently stirred for 15 min, before  
31 145 absorbance measurement at 412 nm (spectrophotometer Carry 100 UV-VIS, Agilent  
32 146 Technologies, USA). The TFS content is calculated using the following equation (Shen, Fang, Gao,  
33 147 & Guo, 2017), with D the dilution factor:

$$144 \quad 148 \quad [SH]_{(\mu\text{mol SH/g})} = \frac{73,53 D A_{412}}{C_p}$$

### 50 151 2.2.2.3 Surface hydrophobicity

51 152 Surface hydrophobicity is determined in triplicate from fluorescence measurements (Shen et al.,  
52 153 2017), with the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS). Five protein solutions  
53 154 (of 4 mL) were prepared from 0.0025 % to 0.02% w/v in a phosphate buffer (8 mM, pH 7.0). The  
54 155 fluorescence intensity is measured after ANS addition (20 µL), for excitation and emission  
55 156 wavelengths of 390 nm and 470 nm, respectively (spectrophotometer Carry 100 UV-VIS, Agilent

157 Technologies USA). The relative fluorescent intensity (RFI) is determined as  $RFI = (F - F_0)/F_0$ , with  
158 F the fluorescence of the protein-ANS conjugate, and  $F_0$  the fluorescence of the ANS probe alone.  
159 The surface hydrophobicity is calculated from the initial slope of RFI versus protein  
160 concentration.

#### 2.2.2.4 Protein profile

162 SDS-Page gel electrophoresis is performed with a Mini-PROTEAN Tetra System, with Mini-  
163 PROTEAN TGX gels and Precision Plus Protein Unstained Standards from Bio-Rad (USA). Protein  
164 suspensions ( $C_p=5 \mu\text{g}/\mu\text{L}$ ) are mixed with an equal volume of 2X Laemmli buffer (5% v/v  $\beta$ -  
165 mercaptoethanol, 40% v/v glycerol, 12.5% v/v Tris-HCl at 0.5 mol/L (pH=6.8), 20% v/v SDS 10%  
166 w/v, 1% v/v of bromophenol blue, and 21.5% v/v distilled water). A volume of 10  $\mu\text{L}$  of sample is  
167 then loaded on each lane of the gel. Migration is performed at 200 V in a running buffer  
168 composed of 1% w/v SDS, Tris buffer (250 mM) and glycine solution (1.92 M, pH=8.3). After  
169 staining with Coomassie brilliant blue R-250 for one hour, gels are thoroughly washed in distilled  
170 water and scanned in a GS-800 Calibrated densitometer (Bio-Rad, USA), before image analysis  
171 with PDQuest 2-D Gel Analysis Software (v 6-2-1).

### 2.2.3 Gelation and gel characterization

174 Protein suspensions are all submitted to a thermal treatment (95 °C for 40 min), preceded or not  
175 by an enzymatic pre-treatment with transglutaminase. Whenever used, transglutaminase is  
176 added to the suspension under magnetic stirring for 5 min. Its concentration is always 0.3wt%,  
177 as this concentration induces the gelation of grinded peanuts suspensions (Basse et al., 2018).  
178 The sample naming is specified in Table 1. It distinguishes samples based on (i) the applied  
179 process (Thermal Treatment, TT or enzymatic + Thermal Treatment, eTT) and (ii) the Ionic  
180 Strength during protein Extraction (E) and (after dialysis) during Gelation (G). Due to the different  
181 dilution factors observed during dialysis, the lowest and highest protein concentrations ( $C_p$ )  
182 slightly differ from one sample to another, as indicate in the table.

#### 2.2.3.1 Rheological characterization

185 Rheological tests are performed with a stress-controlled rheometer Physica MCR 301 (Anton  
186 Paar, Austria) in a Couette cell (sample volume = 25 mL, gap = 1 mm) equipped with a Peltier  
187 temperature controller. Gelation is monitored *in situ* under a constant oscillatory strain  $\gamma = 1\%$



188 and at a constant frequency  $f = 1$  Hz. Prior tests ensured that such low oscillatory strain did not  
189 affect gelation and remain in the linear regime for the final gel characterization. Whatever the  
190 gelation process, the suspension is first maintained for 5 min at 25 °C. For samples submitted  
191 only to a thermal treatment (TT), the temperature was then increased to 95 °C at a rate of  
192 3.5 °C/min, then hold at 95 °C for 40 min, before being decreased to 25 °C at a rate of 3.5 °C/min.  
193 For samples submitted to an enzymatic pre-treatment (eTT), the temperature was increased to  
194 40 °C at 3.5 °C/min, and held for 3 h at 40 °C: at this temperature, the enzymatic activity is about  
195 60% and is stable over time (supplier's data). Then, the temperature evolution follows the TT  
196 procedure. Once gelation is achieved, a frequency sweep is performed at a strain  $\gamma=1\%$ , from  
197  $f=0.01$  Hz to  $f=100$  Hz, followed by a strain sweep from  $\gamma=0.01\%$  to 1000% ( $f=1$  Hz).

### 2.2.3.2 Confocal Laser Scanning Microscopy (CSLM)

200 Confocal images are obtained with a TCS SP8 AOBS inverted confocal microscope (Leica, Solms,  
201 Germany) equipped with a helium-neon laser (458 nm excitation wavelength) and an argon laser  
202 (633 nm excitation wavelength). Protein labelling is performed with the commercially available  
203 chromophore DyLight 488 nm prepared at 0.5 mg/mL (Thermo Fisher Scientific, Waltham, MA,  
204 USA). Fragments of the gels are sampled with a spatula and laid on a 2 $\mu$ L drop of chromophore  
205 before observation.

## 3. Results and discussion

### 3.1 Protein extracts characterization

209 The protein concentration in the freeze-dried protein powders varied from batch to batch, from  
210 35wt% to 49wt% and from 43wt% to 50wt% when extraction was conducted at 0.5 M and 0.8 M,  
211 respectively. In each batch, local heterogeneities were observed, therefore, for each suspension,  
212  $C_p$  is determined based on the protein content of the single batch used for its preparation.

213 The protein composition of the two extracts at 0.5 M and 0.8 M determined by electrophoresis  
214 showed 25 bands, from 8 kDa to 250 kDa, assigned to the different subunits of conarachins and  
215 arachins as depicted in Figure 1. The band at 64 kDa, attributed to conarachins subunits (Toomer,  
216 2017) is 1.6 times more intense in the proteins extracted at 0.5 M (lanes 2-4) than in the ones  
217 extracted at 0.8 M (lanes 5-7). In the opposite, the bands at 22 kDa, 37 kDa and 39 kDa,  
218 attributed to arachins subunits, are 3 to 5 times more intense in the samples extracted at 0.8 M

219 (lanes 5-7) than in the ones extracted at 0.5 M (lanes 2-4). The conarachins/arachins ratio is  
220 therefore ~5-8 times larger in the 0.5M extract than in the 0.8 M extract, which is consistent  
221 with previous results (Monteiro & Prakash, 1994). Considering this large difference, in the  
222 following, the 0.5 M extract will be named the conarachins-rich extract, and the 0.8 M extract  
223 the arachins-rich extract.

224 Thiol group content and surface hydrophobicity are relevant characteristics to assess proteins  
225 ability to form disulfide bond and interact through hydrophobic interaction, respectively. As  
226 conarachins have 2-3 times more sulfur-containing amino acid than arachins (W. L. Brown, 1942;  
227 Wang, 2017), it is expected that the conarachins-rich sample (0.5M extract) contains a higher  
228 amount of thiol groups. Indeed, the thiol content of the 0.5 M extract is  $17.6 \pm 2.3 \mu\text{mol/g}$ , against  
229  $11.5 \pm 2.0 \mu\text{mol/g}$  for the 0.8 M extract, these values being close to the one recently determined  
230 on Peanut Protein Isolate (X. Sun, Jin, Li, Feng, & Liu, 2018). By contrast, surface hydrophobicity  
231 is not significantly different between the two extracts:  $248 \pm 24$  (AU) and  $206 \pm 27$  (AU) for the 0.5  
232 M and 0.8 M extracts, respectively.

233

## 3.2 Gelation and rheological properties

### 3.2.1 Evolution of the rheological properties during gelation

236 In this section, we discuss the rheological evolution of the sample during gelation, focusing only  
237 on the case in which the ionic strength (0.5 M or 0.8 M) imposed for protein extraction is also the  
238 one imposed during the gelation process. The latter consists in either (i) a thermal treatment at  
239  $95 \text{ }^\circ\text{C}$  for 40 min (TT process), or (ii) the same thermal treatment preceded by an enzymatic  
240 pretreatment with Transglutaminase at  $40 \text{ }^\circ\text{C}$  for 3 h (eTT process).

241

242 All samples submitted to the TT process behave as usually observed for globular proteins (Spotti,  
243 Tarhan, Schaffter, Corvalan, & Campanella, 2017): in the initial suspension, the loss modulus  $G''$   
244 is higher than the storage modulus  $G'$ , then both moduli rapidly increase and reach a plateau  
245 with  $G' \approx 10G''$  (Figure 2). When the temperature is decreased back to  $25 \text{ }^\circ\text{C}$ , a further increase of  
246 the moduli occurs, with  $G'_{25^\circ\text{C}} / G'_{95^\circ\text{C}} \approx 2-10$ , whatever the gelation process or the ionic strength.  
247 It is attributed to an entropic effect (Eleya & Turgeon, 2000) and a reinforcement of hydrogen  
248 bonding and intermolecular forces while decreasing the temperature (Chronakis & Kasapis,  
249 1993).

1 251 If the thermal treatment is preceded by an enzymatic pre-treatment (eTT process), the behavior  
2  
3 252 is profoundly different and depends on both the ionic strength and the protein concentration.  
4

5  
6 253 At the lowest protein content ( $C_p \approx 4 \text{ g}/100 \text{ g}$ ), enzymatic pre-treatment itself is not associated  
7  
8 254 with any rheological signature: both  $G'$  and  $G''$  remain constant, with the storage modulus that  
9  
10 255 is almost negligible owing to the liquid-like behavior of the suspension. However, when  
11  
12 256 submitted to the thermal treatment, the protein ability to form a gel is now limited: for the 0.8 M  
13  
14 257 extract, gel formation is inhibited, while for the 0.5 M extract, gelation still occurred but gel  
15  
16 258 strength ( $G' \approx 3 \text{ Pa}$ ) is lower than without pre-treatment ( $G' \approx 6 \text{ Pa}$ ).

17  
18 259 At the highest protein content ( $C_p \approx 11\text{-}13 \text{ g}/100 \text{ g}$ ), both extracts form a gel. For the 0.5 M  
19  
20 260 extract, gelation occurred during the enzymatic pre-treatment, giving rise to very weak gels  
21  
22 261 ( $G' \approx 0.1 \text{ Pa}$ ). The following thermal treatment significantly strengthen the gel, and the final  
23  
24 262 modulus is similar to the one obtained with thermal treatment only ( $G' \approx 300\text{-}450 \text{ Pa}$ ). By contrast,  
25  
26 263 for the 0.8 M extract, gelation is not observed during enzymatic pre-treatment (but a slight  
27  
28 264 increase in  $G'$  possibly suggests the formation of aggregates). Gelation occurred later on during  
29  
30 265 the thermal treatment, but the final modulus remains below the one of samples only submitted  
31  
32 266 to thermal treatment.

33  
34 267  
35  
36 268 All gels showed finally a similar rheological signature, with  $G'/G'' \approx 10$  and a slight moduli  
37  
38 269 dependence towards angular frequency  $\omega$ :  $G' \sim \omega^a$ , with  $0.08 < a < 0.2$  for  $0.06 \text{ rad}\cdot\text{s}^{-1} < \omega < 10 \text{ rad}\cdot\text{s}^{-1}$   
39  
40 270 <sup>1</sup> (results not shown). Strain sweep confirmed that measurements conducted at  $\gamma=1\%$  were well  
41  
42 271 in the linear regime, however, we did not observe any evolution of the limit of linearity with the  
43  
44 272 protein concentration, gelation process or ionic strength (results not shown).

45  
46 273 These first results show that both ionic strength and gelation process play a key role. The 0.5 M  
47  
48 274 extracts (conarachins-rich) exhibit a higher propensity to gel and result in firmer gels than the  
49  
50 275 0.8 M extracts (arachins-rich). The pre-treatment with transglutaminase results in gels with a  
51  
52 276 lower strength, and inhibit gelation at low proteins concentration. In the next sections, we will  
53  
54 277 investigate in details these effects.

55  
56 278

57  
58  
59 279

### 280 3.2.2 Sol-gel transition

1 281 The sample gelation or sol-gel transition, determined as the cross-over between  $G'$  and  $G''$ , is  
2  
3 282 now investigated.

4  
5 283 For suspensions only submitted to thermal treatment, gelation occurred during the temperature  
6  
7 284 ramp, 20 to 30 min after launching the experiments for temperatures ranging from 60 °C to 95 °C.

8  
9 285 For both ionic strengths, the gel point decreases with the protein concentration  $C_p$  (Figure 3, top  
10  
11 286 panels). The higher the  $C_p$ , the shorter the time required for the growing protein aggregates to  
12  
13 287 span the entire volume, as observed for other globular proteins (Tobitani & Ross-Murphy, 1997).

14  
15  
16 288 For suspensions submitted to an enzymatic pre-treatment, the behavior depends on the ionic  
17  
18 289 strength (Figure 3, lower panels).

19  
20 290 For the  $E_{0.5}G_{0.5}eTT$  samples (conarachins-rich), the gelation time depends on the protein  
21  
22 291 concentration. At high concentrations ( $C_p > 6wt\%$ ), most of the samples gelled in the first three  
23  
24 292 hours, during the enzymatic treatment. At low concentrations, ( $C_p < 6wt\%$ ), gelation only  
25  
26 293 occurred later on, during the thermal treatment.

27  
28 294 For the  $E_{0.8}G_{0.8}eTT$  samples (arachins-rich), the scenario is completely different. Although it is  
29  
30 295 known that the transglutaminase activity increases with ionic strength (Kütemeyer, Froeck,  
31  
32 296 Werlein, & Watkinson, 2005), gelation always occurred during the thermal treatment (except for  
33  
34 297 one single case). As arachins contain about 15 times less lysine (transglutaminase substrate) than  
35  
36 298 conarachins (Brown, 1942; Wang, 2017), their reactivity under enzymatic treatment is much  
37  
38 299 lower. During the thermal treatment, the gelation time decreases when the protein  
39  
40 300 concentration increases, and the gelation temperature of the 0.8 M extract is higher, for a given  
41  
42 301 concentration, than the one of the 0.5 M.

43  
44 302 We also observe that the gelation time does not differ significantly whether gelation occurred at  
45  
46 303 the ionic strength imposed for proteins extraction or at a different one under the experimental  
47  
48 304 conditions investigated.

49  
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51 305

### 52 306 3.2.3 Fractal-like scaling of the storage modulus

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54  
55 307 One common pathway for proteins and colloids gelation consists in the fractal aggregation of  
56  
57 308 individual elementary objects eventually forming a three-dimensional network (Ikeda,  
58  
59 309 Foegeding, & Hagiwara, 1999; Wu & Morbidelli, 2001). A rheological signature is the power-law  
60  
61 310 scaling of the plateau storage modulus of the gel with the protein concentration or volume

311 fraction. This has been observed for peanut-protein gels formed by acidification (Bi et al., 2019),  
312 but, as far as we know, never evidenced on heat-set gels of these proteins. Fractal dimensions  
313 can be estimated from the power-law exponent, with the following equation (Shih et al., 1990):

$$G' \propto \varphi^{(3+d)/(3-D)}$$

315 With  $G'$  the storage modulus of the gel (in Pa),  $\varphi$  the volume fraction of the proteins,  $d$  the fractal  
316 dimension of the backbone (between 1.1 and 1.3), and  $D_f$  the fractal dimension of the gel.

317 For samples submitted to a thermal treatment only, the storage modulus indeed follows  $G' \sim C_p^x$   
318 , with  $x=3.4$  (Figure 4). Applying the equation given above, we obtain a fractal dimension  
319  $1.74 < D_f < 1.82$ , suggesting a diffusion-limited cluster aggregation regime (Lin et al., 1989). The  
320 fractal-like scaling is similar for both extracts, however, for a given protein concentration,  
321 conarachins-rich samples (0.5 M extracts) exhibit a higher storage modulus than arachins-rich  
322 samples (0.8 M extracts). This is attributed to the higher sulfur content of conarachins, resulting  
323 in a higher propensity to form disulfide bonds during thermal treatment.

324  
325 For samples submitted to an enzymatic pre-treatment before the thermal treatment, the power-  
326 law scaling of the storage modulus is also found.

327 For the  $E_{0.5}G_{0.5}eTT$  samples, the exponent is  $x = 4.6$ , which corresponds to a fractal dimension  
328  $2.07 < D_f < 2.13$ , characteristic of a reaction limited cluster aggregation regime, during which dense  
329 clusters are formed (Lin et al., 1989). Beside low concentration samples gelled during the thermal  
330 treatment, and high concentration samples gelled during the enzymatic pre-treatment, this does  
331 not apparently affect the final modulus, suggesting that the characteristic scale probed during  
332 oscillatory shear has the same fractal dimension, resulting from the initial enzymatic cross-  
333 linking.

334 For the  $E_{0.8}G_{0.8}eTT$  samples, the range of concentration for which samples gelled is much limited:  
335 all samples with  $C_p > 7\text{wt}\%$  gelled, but none of the samples for  $C_p < 6.1\text{wt}\%$ . For  $6.1\text{wt}\% < C_p < 7\text{wt}\%$ ,  
336 half of the 6 samples investigated gelled, and the other half did not, suggesting a minimum  
337 concentration for gelation  $C_g \approx 6.5\text{wt}\%$ . As the concentration range in which gel are obtained is  
338 reduced, the determination of the fractal exponent is difficult. Still, the data are compatible with  
339 a value of 4.6, as for the  $E_{0.5}G_{0.5}eTT$  samples. The absence of gelation below  $C_g \approx 6.5\text{wt}\%$ , contrarily  
340 to the  $E_{0.5}G_{0.5}eTT$  samples, could result from (i) their lower content in conarachins (rich in sulfur  
341 and lysine, both involved in enzymatic and thermal gelation), and (ii) the higher density of the

342 aggregates formed during enzymatic pretreatment (higher fractal dimension), which occupy less  
1 343 volume but rapidly include most, if not all, of the little amount of conarachins solubilized in these  
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3 344 extracts.  
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7 346 It is worth noticing the different behaviors of proteins that were extracted at a certain ionic  
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9 347 strength and then gelled at a different ionic strength.  $E_{0.5}G_{0.8}TT$  and  $E_{0.8}G_{0.5}TT$  samples exhibit a  
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11 348 modulus which value lays between the one of their counterpart extracted and gelled at the same  
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13 349 ionic strength, e.i. samples  $E_{0.5}G_{0.5}TT$  and  $E_{0.8}G_{0.8}TT$ , respectively. However, if submitted to an  
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15 350 enzymatic pre-treatment, the sample gelation is strongly limited. For  $E_{0.5}G_{0.8}TT$  samples, out of  
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17 351 5 samples for which  $3.1\text{wt}\% \leq C_p \leq 7.7\text{wt}\%$ , only one gelled (for  $C_p \approx 5\text{wt}\%$ ), but exhibited a very weak  
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19 352 modulus ( $G' \approx 1 \text{ Pa}$ ). The change in ionic strength, from 0.5 M during extraction to 0.8 M for  
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21 353 gelation, may have resulted in the partial insolubilization of the conarachins extracted at 0.5 M,  
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23 354 thus reducing the suspension propensity to gel. For  $E_{0.8}G_{0.5}TT$  samples, three samples were  
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25 355 prepared with  $3.1\text{wt}\% \leq C_p \leq 7.7\text{wt}\%$ , and none of them gelled. For  $C_p > 7.7\text{wt}\%$ , gel moduli are  
26  
27 356 slightly higher to the ones observed for the  $E_{0.5}G_{0.5}TT$  samples. This might result from the partial  
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29 357 solubilization of a small fraction of conarachins, initially present in the suspension as insoluble  
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31 358 aggregates, thereby reinforcing the gel.  
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### 360 **3.3 Gels microstructure**

37 361 Confocal images of peanut protein gels structure acquired on the samples produced at high  
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39 362 protein concentration are presented in Figure 5 (samples prepared at low protein concentration  
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41 363 are so weak that they broke in pieces during manipulation). While it is not possible to distinguish  
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43 364 between the two extracts at 0.5 M and 0.8 M, it can be observed that applying an enzymatic  
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45 365 pretreatment gives a much thinner and denser structure, which is coherent with the fractal  
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47 366 dimensions previously determined.  
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## 367 4. Conclusion

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2 368 Peanut proteins extracted at different ionic strength, 0.5 M or 0.8 M, strongly differ in their  
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4 369 conarachins/arachins relative contents. We showed that conarachins, extracted in larger  
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6 370 quantities at 0.5 M, strongly contribute to gel formation and gel elasticity. When submitted to a  
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8 371 thermal treatment, conarachins-rich samples gelled at a lower temperature and if the thermal  
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10 372 treatment is preceded by an enzymatic pre-treatment, their gelation is observed already during  
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12 373 the pretreatment (for high enough protein concentration), which is not the case of the arachin-  
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14 374 rich samples. For a given gelation process, conarachins-rich samples also give stronger gels. This  
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16 375 is attributed to their higher cysteine and lysine contents, two amino-acids involved in protein  
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18 376 cross-linking during thermal or enzymatic treatment. Observations in confocal microscopy  
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20 377 showed that the enzymatic pre-treatment gives much denser and thinner structures. Indeed,  
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22 378 while the fractal dimension of thermally treated samples, estimated from rheological  
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24 379 measurement, is about 1.7, it increases to about 2.1 for enzymatically pre-treated samples. These  
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26 380 values are characteristic of gels formed in the diffusion-limited and reaction-limited cluster  
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28 381 aggregation regimes, respectively. The ionic strength imposed during gelation (in the  
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30 382 investigated range) does not strongly modify the gels properties, except if an enzymatic-pre-  
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32 383 treatment is applied. In that case, gelation is inhibited if an ionic strength different from the  
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34 384 extraction one is imposed, suggesting solubility loss. Ionic strength and transglutaminase pre-  
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36 385 treatment therefore constitute two parameters that can be tuned during food processing to  
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38 386 generate protein gels with different properties.

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### 42 388 CRediT authorship contribution statement

43 389  
44  
45 390 **Benoît Basse:** Conceptualization, Investigation, Formal analysis, Validation, Visualization, Writing  
46  
47 391 - Original Draft. **Véronique Bosc:** Conceptualization, Methodology, Visualization, Project  
48  
49 392 administration, Supervision, Writing - Review & Editing. **Jean-Marc Saiter:** Conceptualization,  
50  
51 393 Methodology, Visualization. **Monique Chan-Huot:** Conceptualization, Project administration,  
52  
53 394 Funding acquisition, Writing - Review & Editing. **Jean-Pierre Dupas:** Resources. **Marie-Noëlle**  
54  
55 395 **Maillard:** Conceptualization, Supervision, Writing - Review & Editing. **Paul Menut:**  
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57 396 Conceptualization, Methodology, Visualization, Project administration, Supervision, Writing -  
58  
59 397 Original Draft, Writing - Review & Editing.

1 399 **Declaration of Competing Interest**

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3  
4 400 The authors declare that they have no known competing financial interests or personal  
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6 401 relationships that could have appeared to influence the work reported in this paper.  
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522 **List of Tables**

1 523 Table 1: Classification and protein concentration range ( $C_p$ , in g/100g) of the different  
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Table 1: Classification and protein concentration range ( $C_p$ , in g/100g) of the different samples.

Ionic Strength	Gelation process	
	Thermal Treatment only	Enzymatic pre-treatment followed by Thermal Treatment
Extraction and gelation at 0.5 M	$E_{0.5}G_{0.5}$ TT $3.0 \leq C_p \leq 13.5$	$E_{0.5}G_{0.5}$ eTT $3.0 \leq C_p \leq 13.5$
Extraction and gelation at 0.8 M	$E_{0.8}G_{0.8}$ TT $3.4 \leq C_p \leq 12.1$	$E_{0.8}G_{0.8}$ eTT $3.4 \leq C_p \leq 12.7$
Extraction at 0.5 M and gelation at 0.8 M	$E_{0.5}G_{0.8}$ TT $3.0 \leq C_p \leq 7.8$	$E_{0.5}G_{0.8}$ eTT $3.1 \leq C_p \leq 7.8$
Extraction at 0.8 M and gelation at 0.5 M	$E_{0.8}G_{0.5}$ TT $3.2 \leq C_p \leq 10.3$	$E_{0.8}G_{0.5}$ eTT $3.2 \leq C_p \leq 9.3$

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531 **List of Figures**

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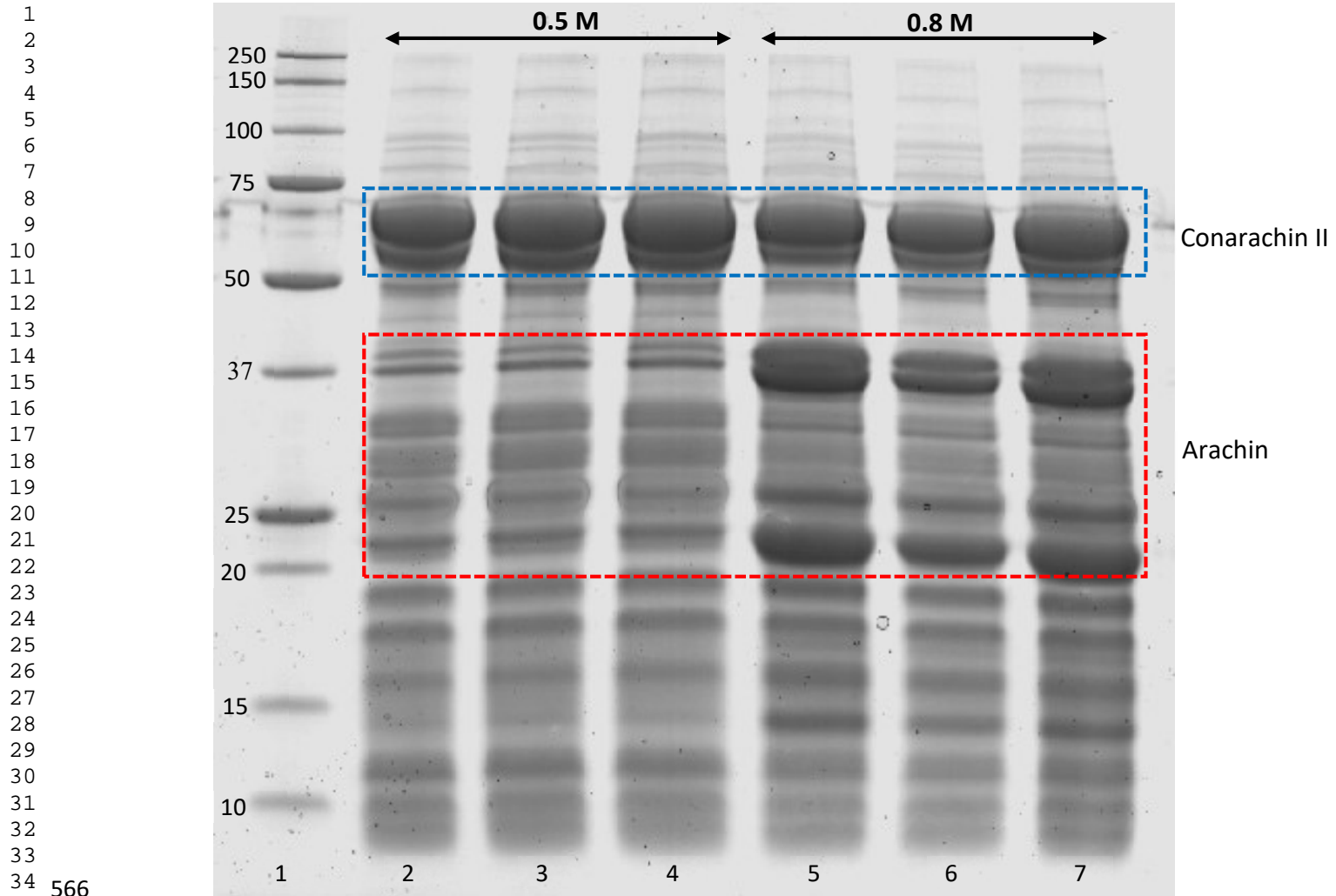
**Figure 1:** SDS-PAGE patterns (in triplicate) of the proteins extracted at 0.5M (lanes 2-4) and 0.8M (lanes 5-7). Standard protein markers are in lane 1 (molecular weights are indicated on the left). Conarachins II subunits (~64 kDa) and arachins subunits (22-42 kDa) are surrounded.

**Figure 2:** Evolution of the storage ( $G'$ , full symbols) and loss ( $G''$ , empty symbols) moduli during gelation, for proteins extracted (and gelled) at ionic strength 0.5 M (blue) and 0.8 M (red). Top panels: thermal treatment alone (TT, circles). Low panels: enzymatic pre-treatment followed by the thermal treatment (eTT, triangles). Low concentration samples ( $C_p = 4$  g/100 g) are on the left and high concentration samples ( $C_p = 11$  or 13 g/100 g) are on the right. Temperature profiles (continuous black lines) are plotted with an axis on the right hand side of the graph.

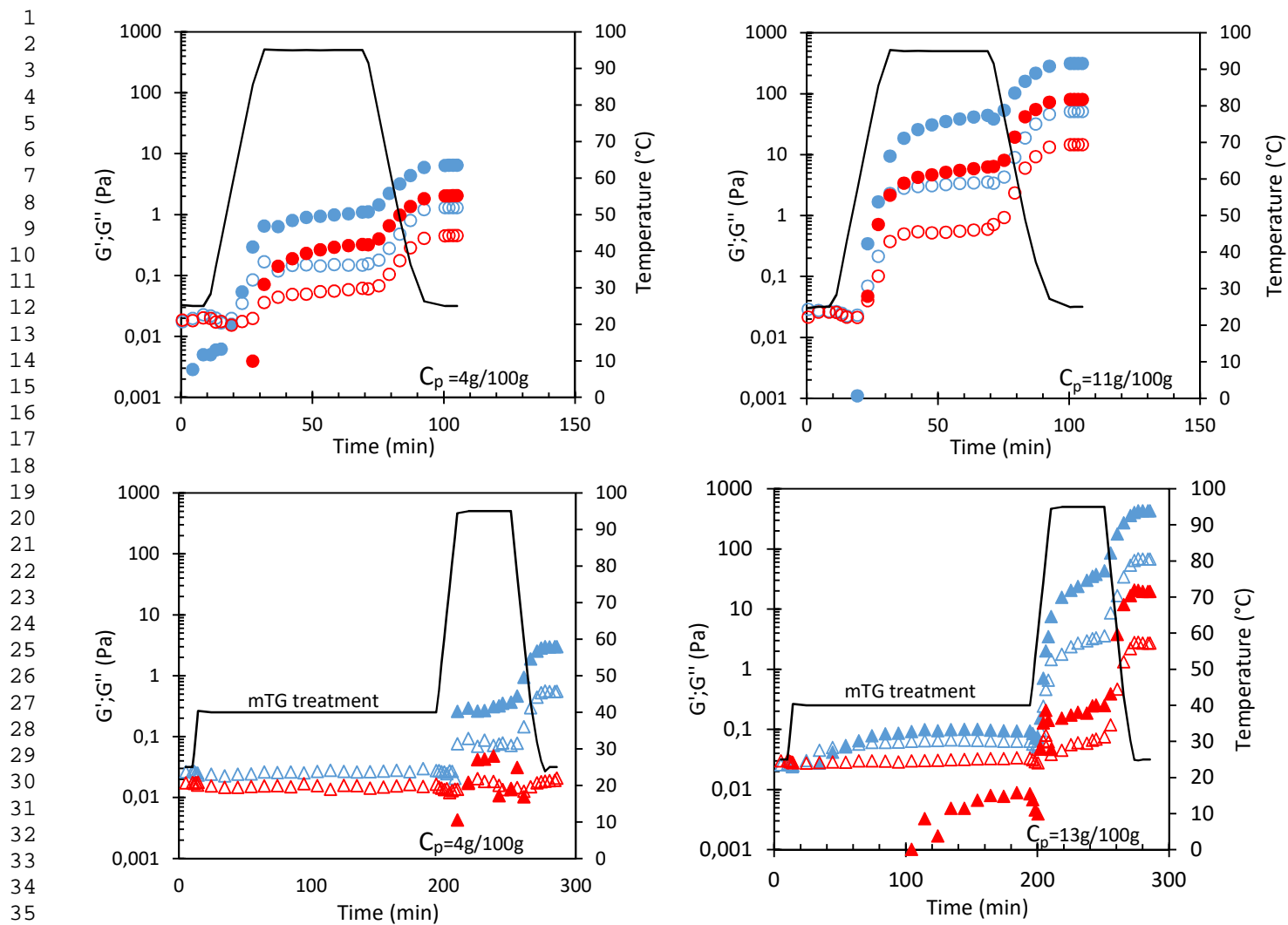
**Figure 3:** Impact of protein concentration on the gelation time (cross-over between the storage  $G'$  and loss  $G''$  moduli), for suspensions submitted to a thermal treatment only (top panel, circles) or to the same thermal treatment preceded by an enzymatic pretreatment (lower panels, triangles). Right panels zoom portions of the left panels during the temperature ramp. Color codes are:  $E_{0.5}G_{0.5}$  (blue),  $E_{0.8}G_{0.8}$  (red),  $E_{0.5}G_{0.8}$  (blue circled with red),  $E_{0.8}G_{0.5}$  (red circled with blue). Color lines are guides to the eye.

**Figure 4:** Storage modulus  $G'$  (Pa) as a function of the protein concentration  $C_p$  (g/100g) for gels obtained by thermal treatment (top panels, circles) and by the same thermal-treatment preceded by an enzymatic pretreatment (lower panels). Color code are the following: blue:  $E_{0.5}G_{0.5}$ , red:  $E_{0.8}G_{0.8}$ , blue circled with red:  $E_{0.5}G_{0.8}$ , red circled with blue:  $E_{0.8}G_{0.5}$ . Continuous lines (left panels) represent power-law fits of the data for samples extracted and gel at the same ionic strength, they are reproduced on the right panels to help the reader (the power-law fit obtained on  $E_{0.5}G_{0.5}TT$  is reproduced in grey in the lower panel). Gelation does not occur at protein concentrations below the vertical dotted lines (in red and blue).

**Figure 5:** Images of peanut protein gels ( $C_p = 12$ g/100g) obtained by CSLM (magnification x40). Proteins are stained in green. Gelation was obtained by Thermal Treatment (TT, top), or by an enzymatic pre-treatment before the thermal treatment (eTT, bottom). Proteins were extracted and gelled at 0.5M (left) or 0.8M (right). Scale bar are 50  $\mu$ m.



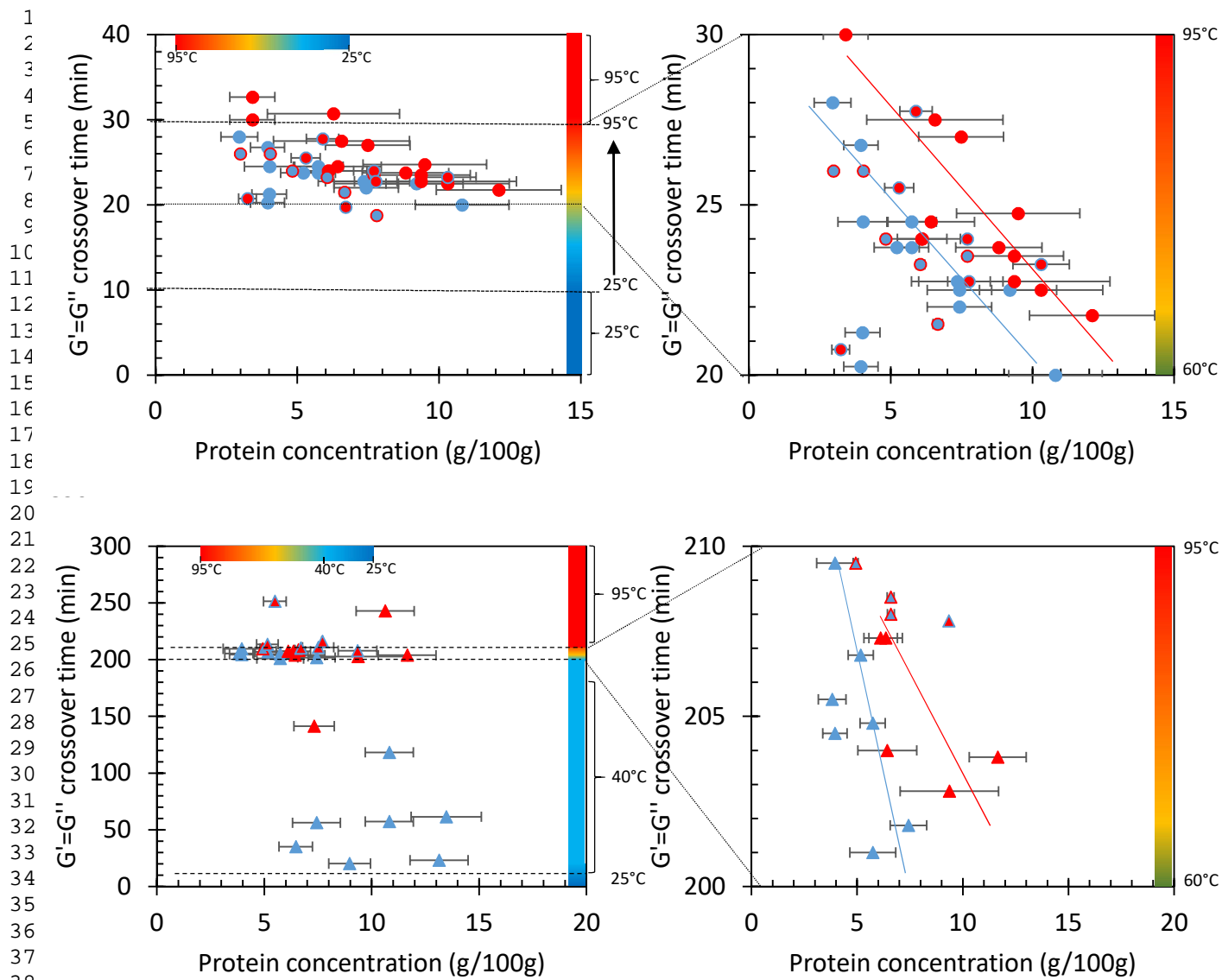
**Figure 1:** SDS-PAGE patterns (in triplicate) of the proteins extracted at 0.5M (lanes 2-4) and 0.8M (lanes 5-7). Standard protein markers are in lane 1 (molecular weights are indicated on the left). Conarachins II subunits (~64 kDa) and arachins subunits (22-42 kDa) are surrounded.



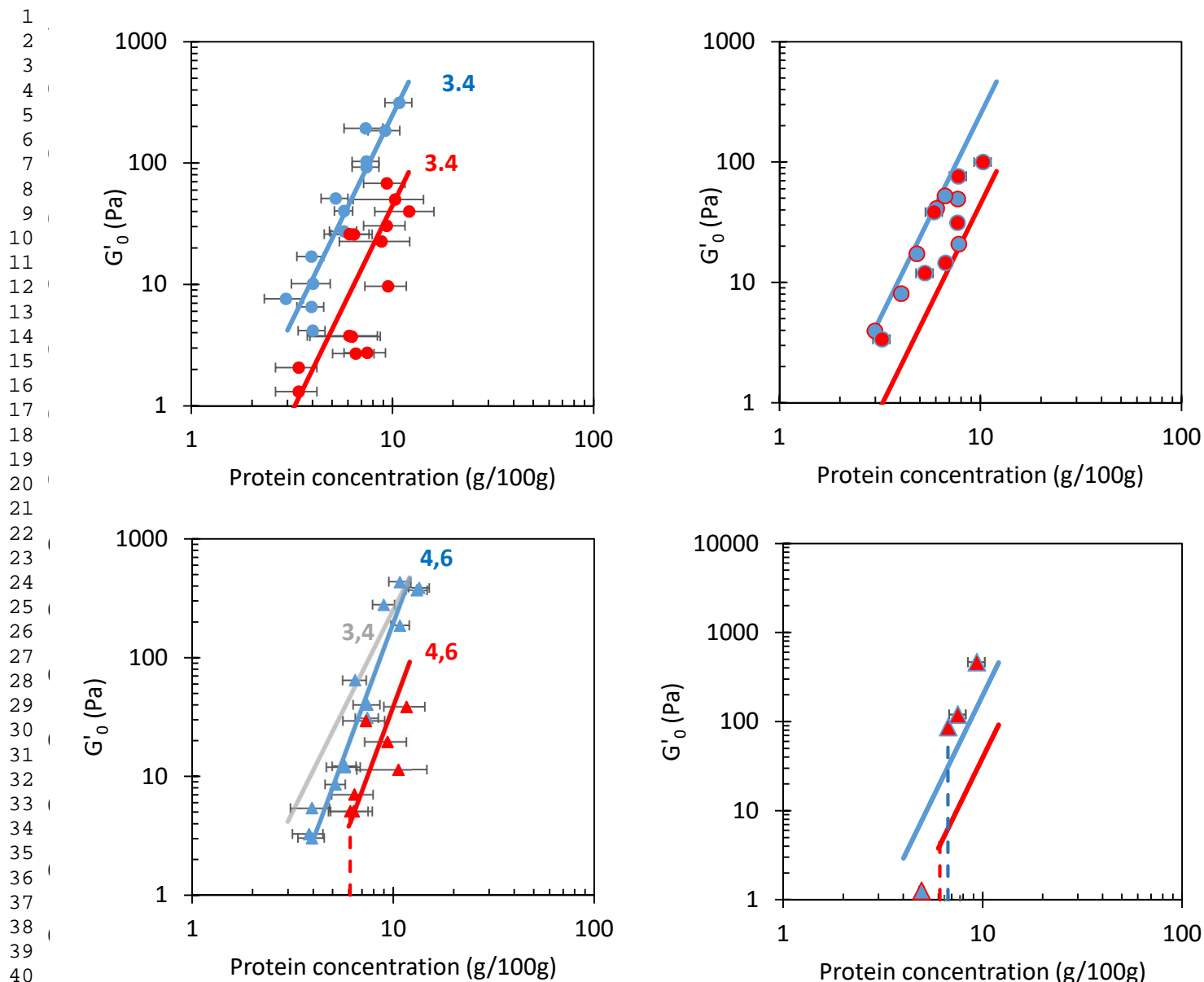
582 Figure 2: Evolution of the storage ( $G'$ , full symbols) and loss ( $G''$ , empty symbols) moduli during gelation, for  
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 584 alone (TT, circles). Low panels: enzymatic pre-treatment followed by the thermal treatment (eTT, triangles). Low  
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 586 on the right. Temperature profiles (continuous black lines) are plotted with an axis on the right hand side of the  
 587 graph.

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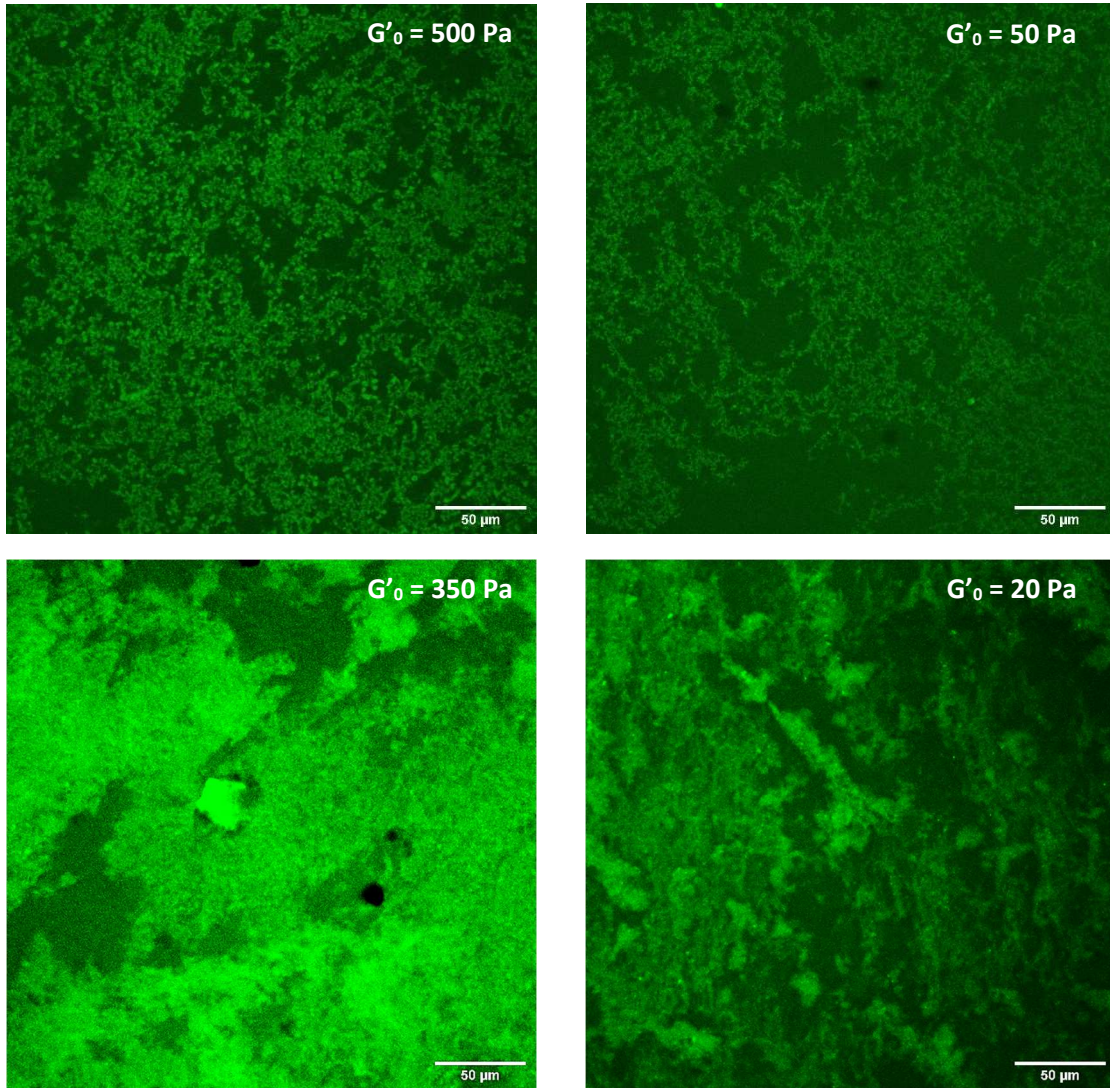
592 **Figure 3:** Impact of protein concentration on the gelation time (cross-over between the storage  $G'$  and loss  $G''$   
 593 moduli), for suspensions submitted to a thermal treatment only (top panel, circles) or to the same thermal  
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 596  $E_{0.8}G_{0.5}$  (red circled with blue). Color lines are guides to the eye.



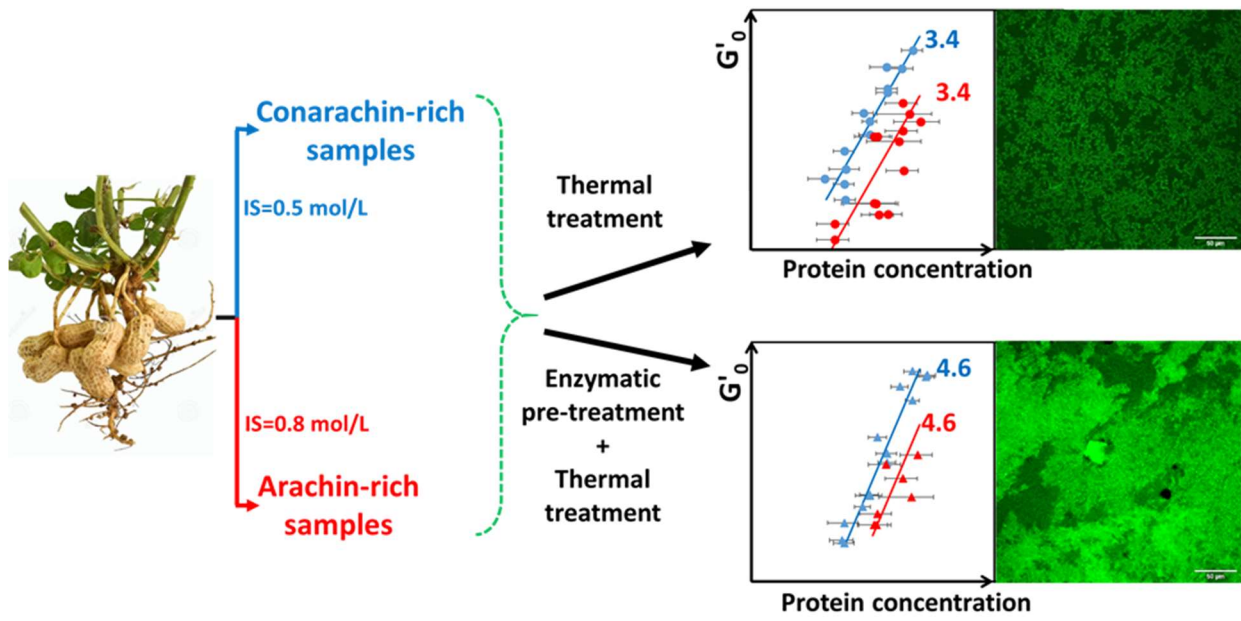
615 **Figure 4:** Storage modulus  $G'$  (Pa) as a function of the protein concentration  $C_p$  (g/100g) for gels obtained by  
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 618 red circled with blue:  $E_{0.8}G_{0.5}$ . Continuous lines (left panels) represent power-law fits of the data for samples  
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 620 power-law fit obtained on  $E_{0.5}G_{0.5}TT$  is reproduced in grey in the lower panel). Gelation does not occur at protein  
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**Figure 5:** Images of peanut protein gels ( $C_p = 12\text{g}/100\text{g}$ ) obtained by CSLM (magnification  $\times 40$ ). Proteins are stained in green. Gelation was obtained by Thermal Treatment (TT, top), or by an enzymatic pre-treatment before the thermal treatment (eTT, bottom). Proteins were extracted and gelled at 0.5M (left) or 0.8M (right). Scale bar are 50 μm.



Declarations of interest: none