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1 **Spray-drying of protein/polysaccharide complexes: dissociation of the**
2 **effects of shearing and heating**

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23 **Abstract**

24 The aim of this study was to dissociate the effect of atomization from that of heating during
25 the spray-drying of Low Methoxyl (LM) pectin/sodium caseinate complexes. The properties
26 of these complexes were studied by measuring turbidity, particle size distribution,
27 zeta-potential, as well as surface hydrophobicity of caseinate within the formed complexes.
28 The results showed that the spraying step had a significant effect on the charge and the size of
29 the complexes. In fact, the application of atomization resulted in the dissociation of
30 caseinate/pectin aggregates especially for high pectin concentrations. Besides, the analysis of
31 the surface hydrophobicity of caseinate indicated that complexation with high concentrations
32 of pectin is able to protect the structure of the protein against heat denaturation. This study
33 allowed a better understanding of the influence of atomization and heat treatment (during the
34 dehydration step) on the molecular interactions within caseinate/pectin complexes.

35

36 **Keywords:** LM pectin, sodium caseinate, atomization, complexes, thermal stability

37 **1. Introduction**

38 In the past few decades, spray-drying technique has been widely used for encapsulation of food
39 ingredients, which focus on protecting and controlling the release of active compounds. During
40 this drying process, the solvent, that is most often water, will be rapidly evaporated, and the
41 active compounds will be entrapped with encapsulating material (Gharsallaoui, Roudaut,
42 Chambin, Voilley, & Saurel, 2007). There are four stages involved in spray drying, namely (i)
43 atomization of the feed solution, (ii) contact of spray with the hot gas, (iii) evaporation of
44 moisture and (iv) particle separation. The loss of free water results in a reduction of water
45 activity and thus extend the shelf life of food products. However, the spray-drying process is
46 accompanied with thermo-mechanical stresses on the active compounds through exposure to
47 high temperatures, distribution of air-water interfaces, shearing, and dehydration (Ben Amara,
48 Eghbal, Degraeve, & Gharsallaoui, 2016). During the spray-drying process, the pressure is
49 mainly from two stages, the stage of atomization (distribution of air-water interfaces, shearing)
50 and the stage of heating (high temperature, dehydration). Atomization is a very important and
51 complex step (Foerster, Gengenbach, Woo, & Selomulya, 2016; Wittner, Karbstein, & Gaukel,
52 2018), which is same for other microencapsulation technologies like spray-cooling or
53 spray-chilling. Heat treatment can easily change the structure of thermal sensitive material,
54 especially for protein. This process may cause reversible or irreversible denaturation of proteins,
55 which involves the disruption and possible destruction of both the secondary and tertiary
56 structures. When proteins denatured, they can lose solubility to aggregation due to the exposure
57 of hydrophobic groups and some bio-functional proteins (enzymes) can lose their biological
58 function due to the loss of the active site.

59 The research concerning the formation of complexes between proteins and polysaccharides

60 has received more and more attention (Devi, Sarmah, Khatun, & Maji, 2017; Li & de Vries,
61 2018). In fact, adding polysaccharides to form protein-polysaccharide complexes can somehow
62 protect protein against denaturation during spray-drying process as shown in our previous works
63 (Ben Amara, Eghbal, Degraeve, & Gharsallaoui, 2016; Ormus, Oulahal, Noël, Degraeve, &
64 Gharsallaoui, 2015).

65 Sodium caseinate is a kind of milk proteins containing α_{s1} -, α_{s2} -, β -, and κ -caseins. These four
66 proteins have a strong tendency to associate with each other to form supra-molecular aggregates
67 (Surh, Decker, & McClements, 2006). Pectin is a natural polysaccharide found as a component
68 of cell walls in fruits and some roots, which is widely used in the food industry for its thickening
69 and gelling properties. It is composed by a chain of α -(1-4)-linked D-galacturonic acid.
70 According to the degree of methyl esterification, pectin can be classified as high-methoxyl (HM)
71 and low-methoxyl (LM) pectin. Compared to HM pectin, LM pectin requires less sugar to form
72 gels, and can form gels in a wider range of pH (from 2.6 to 7.0). The system of pectin and
73 caseinate is extensively investigated since over twenty years (Cheng & McClements, 2016;
74 Matia-Merino, Lau, & Dickinson, 2004; Turgeon, Schmitt, & Sanchez, 2007) and it was recently
75 explored in our previous studies (Eghbal, Degraeve, Oulahal, Yarmand, Mousavi, & Gharsallaoui,
76 2017; Eghbal, Yarmand, Mousavi, Degraeve, Oulahal, & Gharsallaoui, 2016). According to these
77 studies, complex coacervation of caseinate and LM pectin could happen at pH 3. At this pH,
78 positively-charged caseinate and negatively-charged pectin can form insoluble complexes due to
79 electrostatic interactions. A greater understanding of how caseinate interact with pectin is
80 essential to control structure, functionality and applicability of this system.

81 In this study, we separated the atomization process and heating process in spray-drying by
82 dissociating the atomizer from spray-drier and replacing heating process with water bath heat

83 treatment. To the best of our knowledge, the separate effects of atomization and heating steps
84 during spray-drying on the properties of protein/polysaccharide complexes were not reported in
85 literature. Through testing the physicochemical properties of caseinate/pectin complexes before
86 and after atomization and heat treatment, we hoped to investigate the effect of atomization and
87 heat treatment on the formation of protein/polysaccharide complexes.

88

89 **2. Materials and methods**

90 **2.1. Materials**

91 LM pectin (Unipectine™ OF 305 C SB) was purchased from Cargill (Baupre, France). The
92 degree of esterification was from 22% to 28% and the degree of acetylation was from 20% to
93 23%. Sodium caseinate was purchased from Fisher Scientific (United Kingdom). Protein content
94 in sodium caseinate determined by the Kjeldahl method was 93.20% (nitrogen conversion factor
95 N=6.38). Analytical grade imidazole (C₃H₄N₂), acetic acid, sodium hydroxide (NaOH),
96 hydrochloric acid (HCl), 8-Anilino-1-naphthalenesulfonic acid (ANS) were purchased from
97 Sigma-Aldrich Chimie (St Quentin Fallavier, France).

98 **2.2. Solution preparation**

99 Imidazole-acetate buffer solutions (5 mM) were prepared by dispersing imidazole and
100 acetic acid into distilled water and then adjusting the pH to 3.0. The stock solutions were
101 prepared by dispersing LM pectin and sodium caseinate powders to unadjusted imidazole-acetate
102 buffer and stirring for at least 2 h at room temperature, and then adjusting the pH to 3.0. The pH
103 was adjusted by using HCl (0.1 or 1.0 M) or NaOH (0.1 or 1.0 M). Complexes solutions were
104 prepared by mixing the stock solutions and then diluted to the desired concentration by
105 imidazole-acetate buffer (pH 3.0). The final sodium caseinate concentration was constant at 5.0
106 g/L, and the LM pectin concentration was 0.5, 2.0 and 6.0 g/L. After mixing, the complexes
107 solutions were stirred for at least 2 h at room temperature and the pH was checked again.

108 **2.3. The atomization process**

109 The atomizer (Mini spray-dryer B-290) was manufactured by BUCHI (Switzerland). The
110 atomizer nozzle was disassembled to prepare the complex solutions after atomization. The

111 nozzle consists of three inputs: an air inlet and two inputs for the liquid as well as an outlet. An
112 atomizer pump that set at a flowrate of 0.5 L/h was used to feed the nozzle with the previously
113 prepared complex solutions. In order to study the effect of the shearing applied during the
114 spray-drying process, the sodium caseinate/LM pectin complex mixtures were subsequently
115 sprayed at a rate of 0.5 L/h through the nozzle of the atomizer (complex after spraying) and
116 recovered in containers until the tests are carried out. All the atomization experiments were
117 carried at room temperature, the suspensions of complexes before and after spraying were also
118 maintained at room temperature.

119 **2.4. Heat treatment**

120 During spray-drying, the suspensions of complexes are exposed to high temperatures during
121 a very short time, which makes it possible to dry the sprayed micro-droplets rapidly. For
122 separately studying the effect of the heat treatment, the various mixtures obtained (in 10 mL
123 tubes) were heated in a water bath at 80 °C for time intervals set at 1 min (T1), 2 min (T2), 3 min
124 (T3) and 4 min (T4). Complexes that underwent heat treatment were compared with unheated
125 complexes (T0).

126 **2.5. Turbidity measurement**

127 The turbidity of all complexes suspensions at pH 3 were measured by using a UV/Vis
128 spectrophotometer (Jenway 3705, Villepinte, France) at 600 nm in plastic cuvettes (1 cm path
129 length). Each suspension was diluted 3 to 6 times with imidazole-acetate buffer at pH 3 until the
130 optical density value of each diluted suspension was less than 1.5. The solution of (5.0 g/L)
131 sodium caseinate was used as a control during the measurements, and every suspension was
132 analyzed at least three times.

133 **2.6. Particle size distribution**

134 Particle size distributions of sodium caseinate/LM pectin complexes were assessed by a
135 laser diffraction instrument (Mastersizer 3000, Malvern Instruments, Malvern, UK). To avoid
136 multiple scattering effects, the complexes were injected into the measurement chamber where it
137 was diluted with imidazole-acetate buffer (5 mM; pH 3) prior to the measurements. The
138 complexes were continuously stirred throughout the measurement to ensure the samples were
139 homogeneous. The volume mean particle diameter (D_{43}) was calculated by the software from the
140 three injections of three separate samples with five readings per sample.

141 **2.7. Zeta potential measurement**

142 The electric charges (ζ -potential) of sodium caseinate/LM pectin complexes were
143 determined using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). If necessary, the
144 samples were diluted with pH 3 imidazole-acetate buffer. The mean ζ -potential (ZP) values (\pm SD
145 (standard deviation)) were obtained from the instrument, standard deviation of measurements
146 that are repeated three times for each suspension.

147 **2.8. Measurement of surface hydrophobicity**

148 For the surface hydrophobicity study, the 8-anilino-1-naphthalenesulfonic acid (ANS)
149 fluorescent probe was used (Bi, Tang, Gao, Jia, & Lv, 2016). For each suspension studied,
150 increasing volumes of the probe (20 mg/L) ranging from 10 to 30 μ L were added to 4.0 ml of the
151 suspension. The maximum fluorescence intensity due to the binding of the ANS molecules to the
152 hydrophobic sites of the protein was measured by carrying out a scanning of the wavelength
153 between 420 nm and 650 nm for an excitation wavelength of 380 nm. The excitation and
154 emission bandwidths were set at 5.0 nm. The scan rate was 500 nm/min. Fluorescence intensity
155 measurements were performed with a LS 55 spectrofluorometer (PerkinElmer, Courtaboeuf,

156 France). All measurements were performed at room temperature using a quartz cuvette (4 faces,
157 1 cm path length). The addition of the probe was achieved until no increase of the fluorescence
158 intensity was observed. The maximum fluorescence intensity was plotted against the
159 concentration of ANS. The initial slope of the curve of maximum fluorescence intensity
160 (arbitrary unit, U.A) versus ANS concentration (mg/L) was calculated and used as an index of
161 surface hydrophobicity (S_0). For each suspension, the test was repeated three times.

162 **2.9. Statistical analysis**

163 All assays were measured at least in triplicate. Means and standard deviations were
164 calculated and differences between means were determined with the Least Significant Difference
165 (LSD) test at $p < 0.05$ significance level (Statgraphics Centurion XV).

166

167 **3. Results and discussion**

168 **3.1. Effect of atomization on the properties of caseinate/pectin complexes**

169 The effect of atomization (shearing) on the properties of caseinate/pectin complexes was
170 studied at three different ratios (sodium caseinate concentration kept constant: 5.0 g/L; LM
171 pectin concentration changed: 0.5, 2.0 or 6.0 g/L). Before atomization, the turbidity of
172 complexes increased (Fig. 1a) with increasing pectin concentration while the average size
173 reached a peak (Fig. 1b). This indicated that, with addition of pectin, caseinate could combine
174 more and more pectin to form complexes leading to a growing size until a pectin concentration
175 of 2 g/L and turbidity increase. When pectin amount continued to increase, the particle size
176 decreased leading to the formation of numerous small complexes resulting in the still increase of
177 turbidity.

178 As shown in Fig. 2a, the ζ -potential values of complexes changed from positive to negative
179 with increasing pectin concentration, due to the constant amount of positively charged caseinate
180 molecules neutralized by the increase amount of negatively-charged pectin. At the low (0.5 g/L)
181 and high (6.0 g/L) concentrations of pectin, the caseinate/pectin complexes were highly charged,
182 and at intermediate (2.0 g/L) concentration of pectin, the complexes were more neutrally charged.
183 These results also confirmed that the size decrease at high (6.0 g/L) concentration of pectin was
184 due to electrostatic repulsions. There was a slight decrease of the protein hydrophobicity only at
185 high pectin concentration (6.0 g/L) (Fig. 2b), which means that hydrophobic sites of caseinate
186 would be masked by the pectin chains.

187 After atomization, the turbidity of complexes was almost constant at low (0.5 g/L) and
188 intermediate (2.0 g/L) concentrations of pectin. At high concentration (6.0 g/L) of pectin, the
189 turbidity of complexes significantly decreased ($p < 0.05$) after atomization (Fig. 1a). The average

190 size of complexes significantly decreased ($p < 0.05$) at all the 3 tested ratios (Fig. 1b), which is in
191 agreement with other published studies (Serfert, Schröder, Mescher, Laackmann, Rätzke, Shaikh,
192 et al., 2013). The partial dissociation of complexes was due to shear forces during their passage
193 in the nozzle and an increase in their overall charge favoring electrostatic repulsions, especially
194 at high concentration (6.0 g/L) of pectin (Fig. 2a). In addition, there was a significant increase of
195 the hydrophobicity S_0 (particularly at 2.0 g/L and 6.0 g/L of pectin), which could indicate
196 molecular rearrangements and unfolding of the structure of the protein caused by exposure of
197 hydrophobic sites (Fig. 2b).

198 **3.2. Effect of heat treatment on the properties of caseinate/pectin complexes**

199 Heat treatment had a significant effect on the turbidity and the size of the complexes (Fig. 3).
200 With the increase of the heat treatment time, the turbidity value of complexes increased ($p < 0.05$)
201 from 0.98 ± 0.05 to $2.87 \pm 0.28 \text{ cm}^{-1}$ at the lower concentration of pectin. It was almost constant
202 ($\sim 4.22 \text{ cm}^{-1}$) at the intermediate concentration of pectin. However, at the higher concentration of
203 pectin, the turbidity value of complexes increased ($p < 0.05$) from $5.29 \pm 0.09 \text{ cm}^{-1}$ to $6.78 \pm$
204 0.04 cm^{-1} until the 4th min where we had a decrease up to $6.05 \pm 0.17 \text{ cm}^{-1}$ (Fig. 3a).

205 As shown in Fig. 3b, the size of complexes formed at low concentration of pectin (0.5 g/L)
206 was constant as a function of heating time ($55.77 \pm 4.53 \text{ }\mu\text{m}$). Combined with the increased
207 turbidity (Fig. 3a), it can be concluded that heat treatment caused the formation of more
208 numerous particles which had the same size as the caseinate/pectin complexes at low pectin
209 concentration. At intermediate concentration of pectin (2.0 g/L), the size increased from $264.17 \pm$
210 26.36 to $380.80 \pm 35.05 \text{ }\mu\text{m}$ ($p < 0.05$) with increase of heat treatment time. Combined with the
211 stable turbidity (Fig. 3a), it clarified that heat treatment caused the aggregation of complexes at
212 this pectin concentration. The most interest part was the significant decrease ($p < 0.05$) of

213 particle size from 220.46 ± 38.08 to 104.16 ± 29.62 μm at high concentration of pectin (6.0 g/L),
214 with increase of heating time. This result indicated that heat treatment caused dissociation of
215 aggregates. This also explained the decrease of turbidity after 4 min of heating time (Fig. 3a).
216 High concentration of LM pectin may have induced bridging flocculation of protein aggregates
217 (Jones, Lesmes, Dubin, & McClements, 2010). It can be assumed that, after heating treatment,
218 bridging flocculation was suppressed.

219 At low concentration of pectin, slight decrease ($p < 0.05$) in ζ -potential (Fig. 4a) and
220 increase in surface hydrophobicity (Fig. 4b) indicated that the structure of complexes changed.
221 Anionic groups of pectin and hydrophobic sites in caseinate molecules could be exposed
222 following heat treatment. The change of the electrostatic charge from positive to negative and a
223 slight increase in the hydrophobicity at intermediate concentrations of pectin indicated that the
224 molecular rearrangement allowed to “protect” the protein from heat denaturation. At high
225 concentration of pectin, surface hydrophobicity was almost constant as the increasing time,
226 which confirmed the protective effect of pectin against heat treatment. According to Jones,
227 Decker and McClements (2010), the ability of LM pectin to increase the thermal denaturation
228 temperature may be due to differences in the electrostatic interactions between carboxyl groups
229 on the pectin molecules and charged groups on the folded and unfolded protein surfaces.

230 **3.3. Combined effect of atomization and heat treatment on the properties of complexes**

231 To simulate the whole spray-drying process, the caseinate/pectin complexes were atomized
232 and subsequently heated. Combined effect of atomization and heat treatment was evaluated by
233 measuring the properties of atomized complexes treated by heating at 80 °C water bath for 0 to 4
234 min.

235 As shown in Fig. 5, with increasing heat treatment duration, at low concentration of pectin

236 (0.5 g/L), the turbidity of atomized complexes slightly increased ($p < 0.05$) from 1.05 ± 0.17
237 cm^{-1} to $2.08 \pm 0.03 \text{ cm}^{-1}$ and the particle size was constant ($44.52 \pm 5.73 \mu\text{m}$). The increase in
238 number of particles could be due to the aggregation of free or partially complexed caseinate
239 molecules. Compared to the properties of caseinate/pectin complexes directly treated by heating
240 without atomization at low pectin concentration (Fig. 3), the atomized complexes had a smaller
241 size and the turbidity of atomized complexes as function of heat treatment duration increased
242 more slightly.

243 At intermediate pectin concentration (2.0 g/L), with the increase of heating duration,
244 compared to the stable turbidity of caseinate/pectin complexes without atomization (Fig. 3a),
245 heat treatment caused the slight decrease ($p < 0.05$) in turbidity of atomized complexes from 4.42
246 $\pm 1.00 \text{ cm}^{-1}$ to $2.93 \pm 0.12 \text{ cm}^{-1}$ (Fig. 5a), and significant increase ($p < 0.05$) in particle size from
247 115.70 ± 34.17 to $379.22 \pm 24.72 \mu\text{m}$ (Fig. 5b). Thus, we can assume that the association
248 between small complexes could be accentuated by electrostatic charge neutralization (Fig. 5c).
249 Compared to the caseinate/pectin complexes directly treated by heating without atomization,
250 atomized complexes formed at intermediate pectin concentration were more sensitive to heat
251 treatment.

252 Atomized complexes formed at high pectin concentration (6.0 g/L) showed a constant
253 particle size ($32.61 \pm 4.62 \mu\text{m}$) and an increased turbidity from $3.85 \pm 0.06 \text{ cm}^{-1}$ to 8.97 ± 0.97
254 cm^{-1} with the increase of heat treatment duration (Fig. 5). Similar results were also found in our
255 previous research (Amara, Eghbal, Degraeve, & Gharsallaoui, 2016). The increase of turbidity
256 could be due to thermal dissociation of aggregates, which was already deconstructed by shearing,
257 and formation of new complexes with free pectin chains. Highly negatively charged complexes
258 could be protected from aggregation by electrostatic repulsions (Fig. 5c).

260 **4. Conclusions**

261 In this study, caseinate/pectin complexes were prepared at pH 3 at three different
262 caseinate/pectin ratios. To evaluate the separate effect of mechanical and thermal steps on the
263 complexes during the spray-drying process, atomization and heat treatment were dissociated and
264 applied to the caseinate/pectin complexes. The properties like turbidity, ζ -potential, particle size
265 and surface hydrophobicity of complexes were measured.

266 The results indicated that atomization caused dissociation of aggregates into smaller size
267 complexes at all pectin concentrations. At low concentration of pectin, heat treatment caused the
268 aggregation of excess caseinate. At intermediate concentration of pectin, heat treatment
269 intensified aggregation of complexes. At high concentration of pectin, heat treatment favored the
270 dissociation of the aggregates. Complexes after atomization were more sensitive to heat
271 treatment, but a high concentration of pectin could protect the complexes against heat
272 aggregation. These results could help to develop new delivery systems based on spray-dried
273 protein/polysaccharide complexes.

274

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- 332

333 **Figure Captions**

334 **Fig. 1.** Effect of atomization on turbidity (a) and particle size (b) of caseinate/pectin complexes
335 at three different concentrations of pectin (the concentration of caseinate was kept constant at 5.0
336 g/L; pectin concentrations was varied from 0.5 to 6.0 g/L; pH 3).

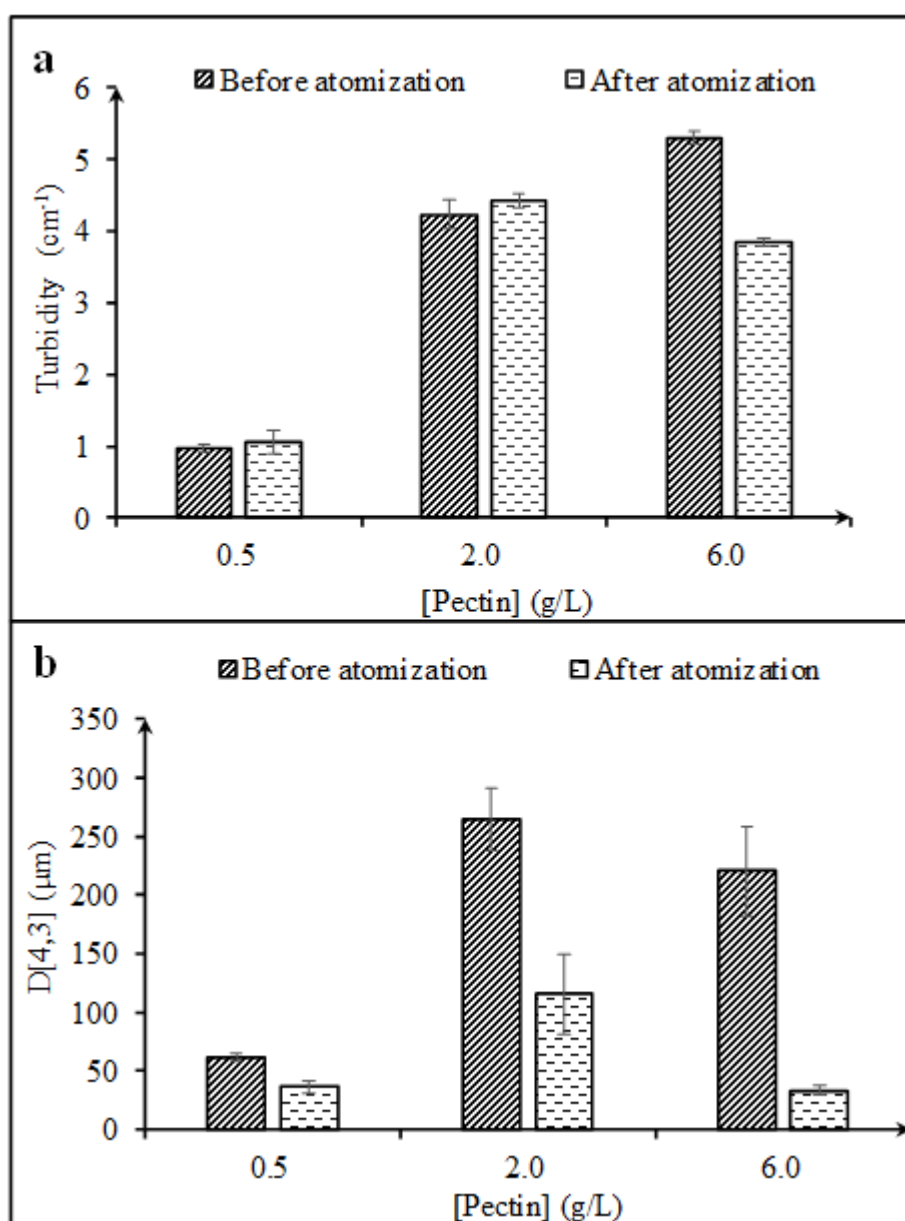
337 **Fig. 2.** Effect of atomization on ζ -potential (a) and surface hydrophobicity (b) profiles of
338 caseinate/pectin complexes at three different concentrations of pectin (the concentration of
339 caseinate was kept constant at 5.0 g/L; pectin concentrations was varied from 0.5 to 6.0 g/L; pH
340 3).

341 **Fig. 3.** Turbidity (a) and particle size (b) of caseinate/pectin complexes at three different
342 concentrations of pectin as function of heat treatment duration from 0 to 4 min at 80 °C (**T0**:
343 unheated control, **T1**: 1 min, **T2**: 2 min, **T3**: 3 min, **T4**: 4 min; the concentration of caseinate was
344 kept constant at 5.0 g/L; pectin concentrations was varied from 0.5 to 6.0 g/L; pH 3).

345 **Fig. 4.** ζ -potential (a) and surface hydrophobicity (b) of caseinate/pectin complexes at three
346 different concentrations of pectin as function of heat treatment duration from 0 to 4 min at 80 °C
347 (**T0**: unheated control, **T1**: 1 min, **T2**: 2 min, **T3**: 3 min, **T4**: 4 min; the concentration of
348 caseinate was kept constant at 5.0 g/L; pectin concentrations was varied from 0.5 to 6.0 g/L; pH
349 3).

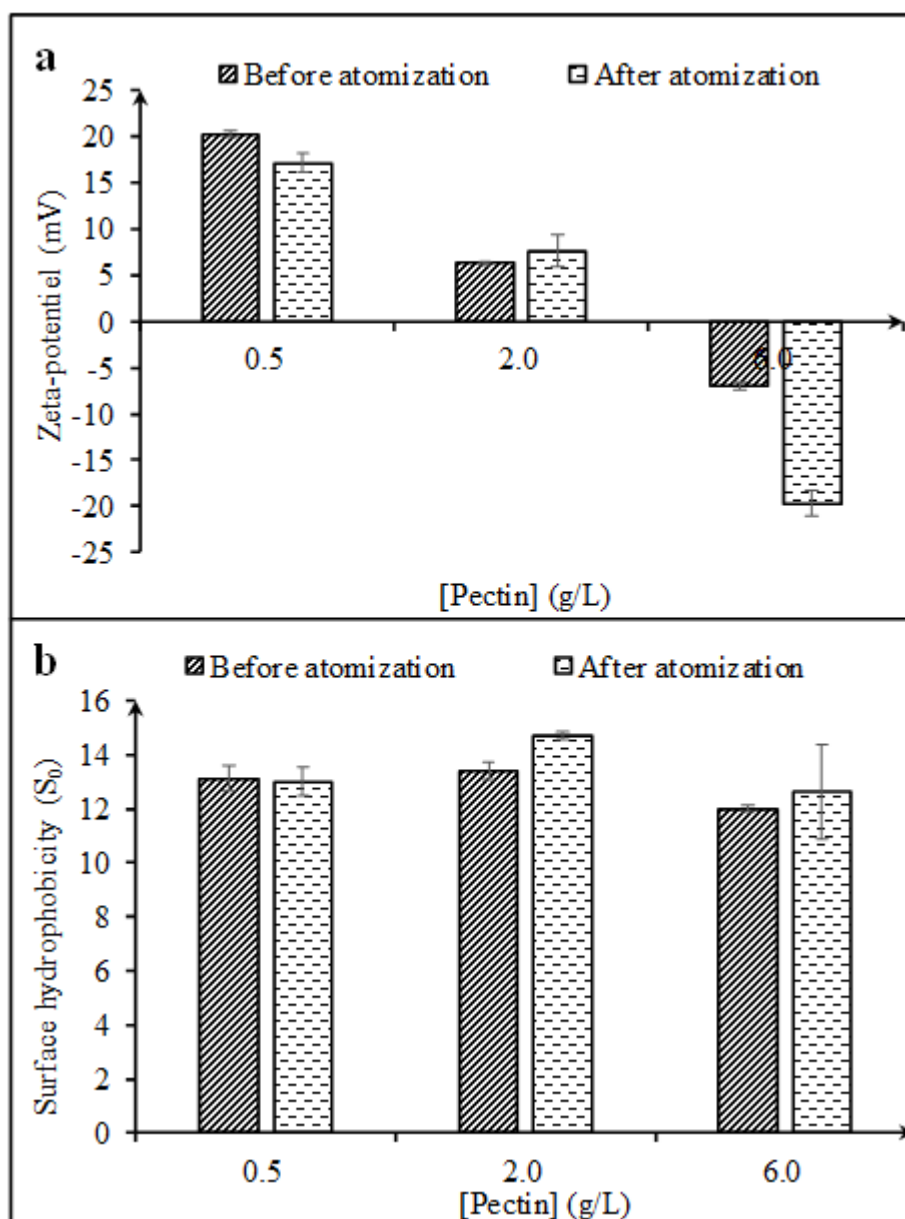
350 **Fig. 5.** Combined effect of atomization and heat treatment (80 °C) on turbidity (a), particle size
351 (b) and ζ -potential (c) of caseinate/pectin complexes at three different concentrations of pectin
352 (**T0**: unheated control, **T1**: 1 min, **T2**: 2 min, **T3**: 3 min, **T4**: 4 min; the concentration of

353 caseinate was kept constant at 5.0 g/L; pectin concentrations was varied from 0.5 to 6.0 g/L; pH
354 3).
355



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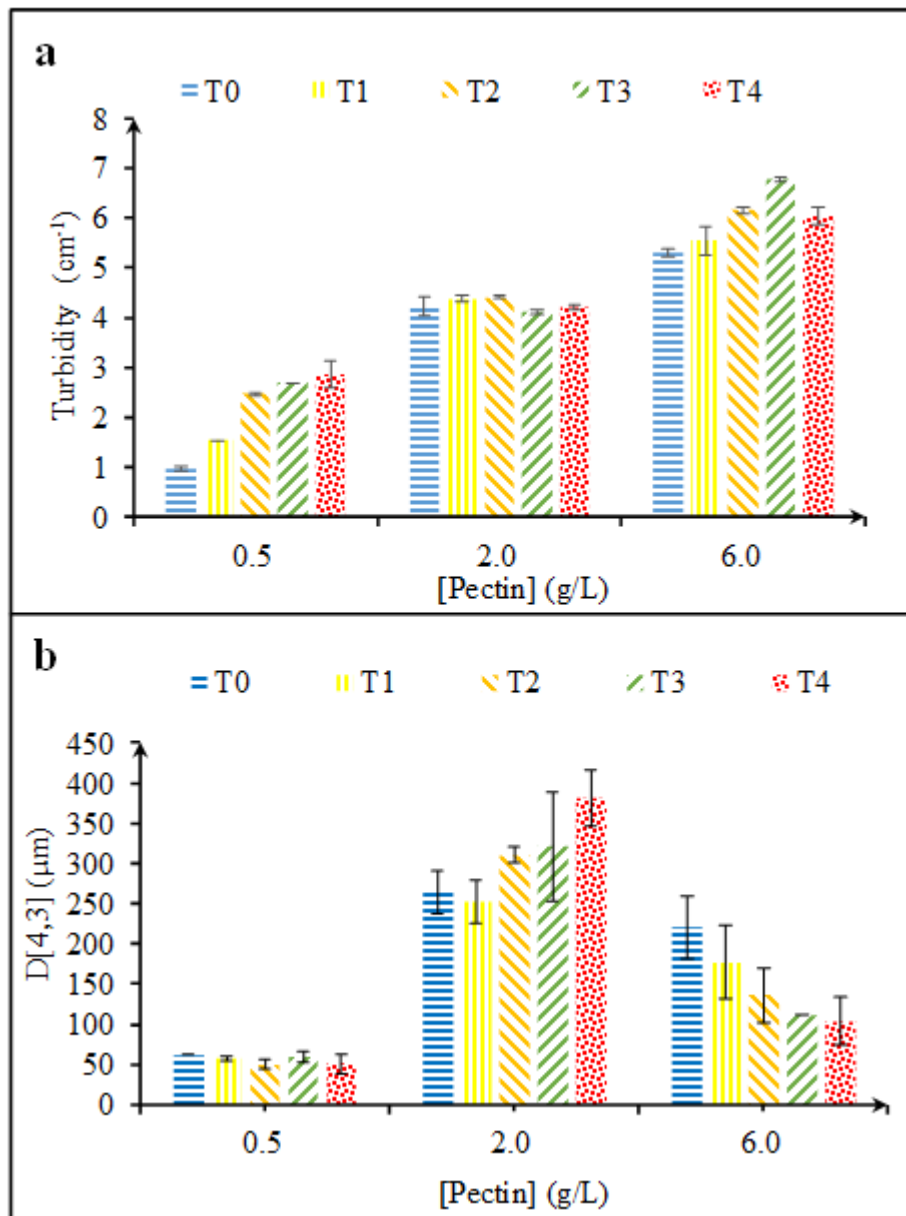
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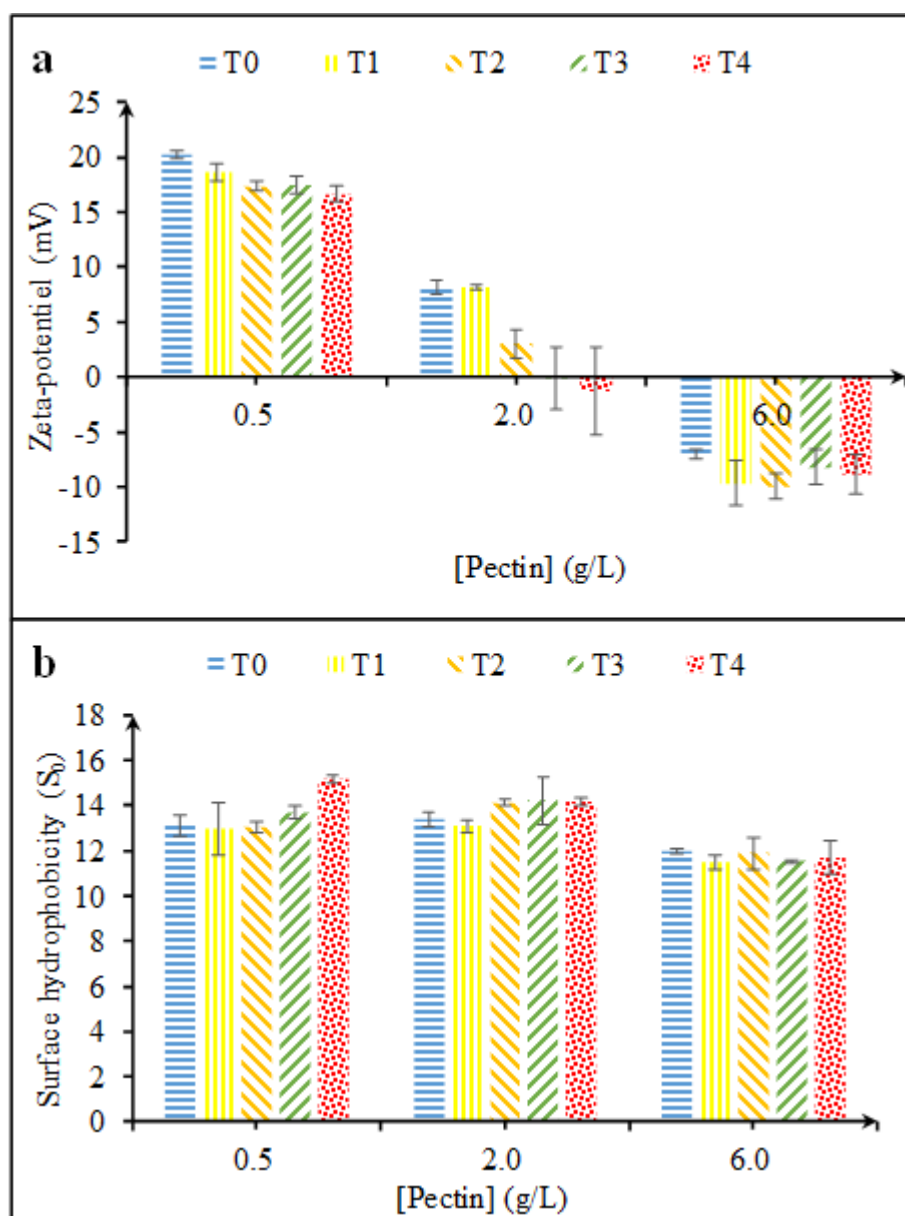
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362 **Fig. 3**



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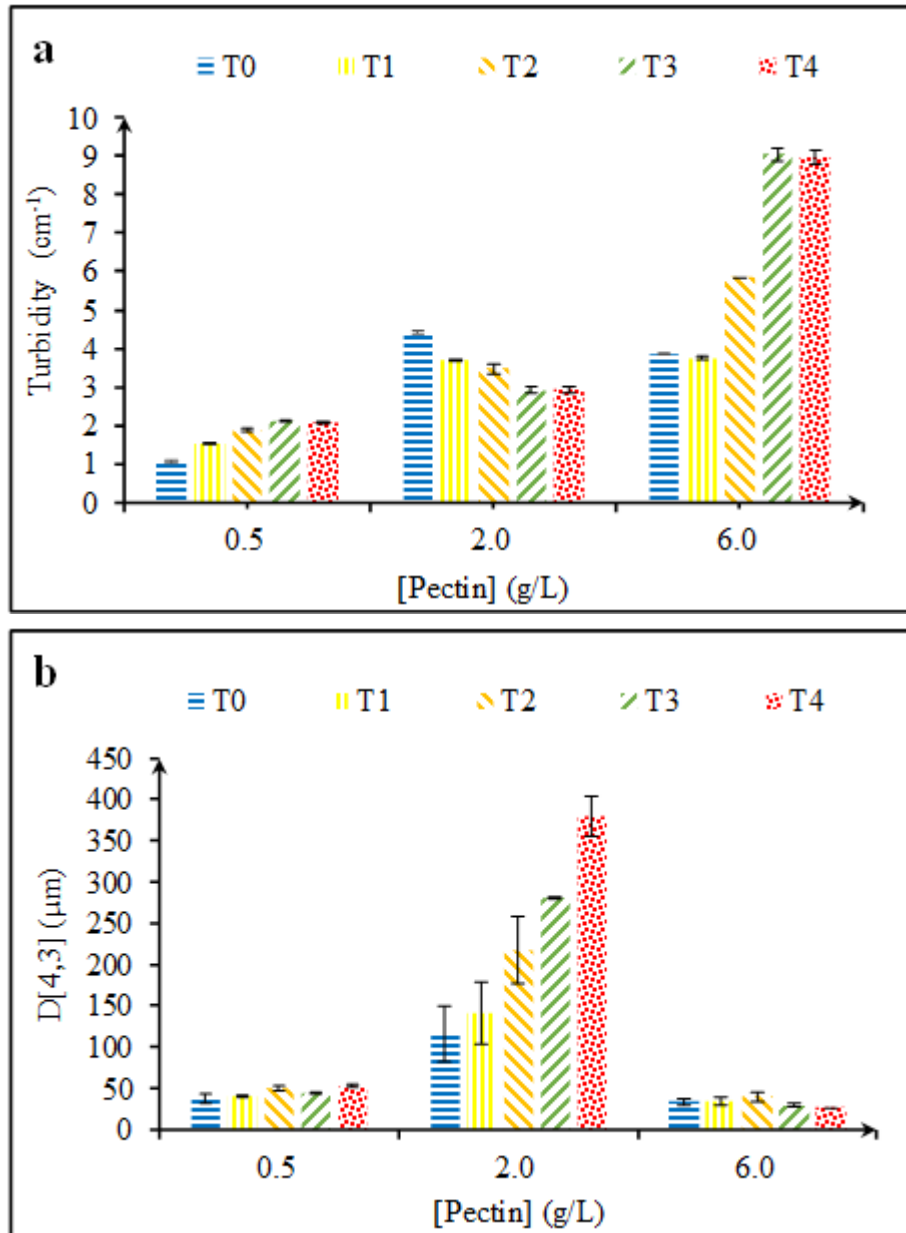
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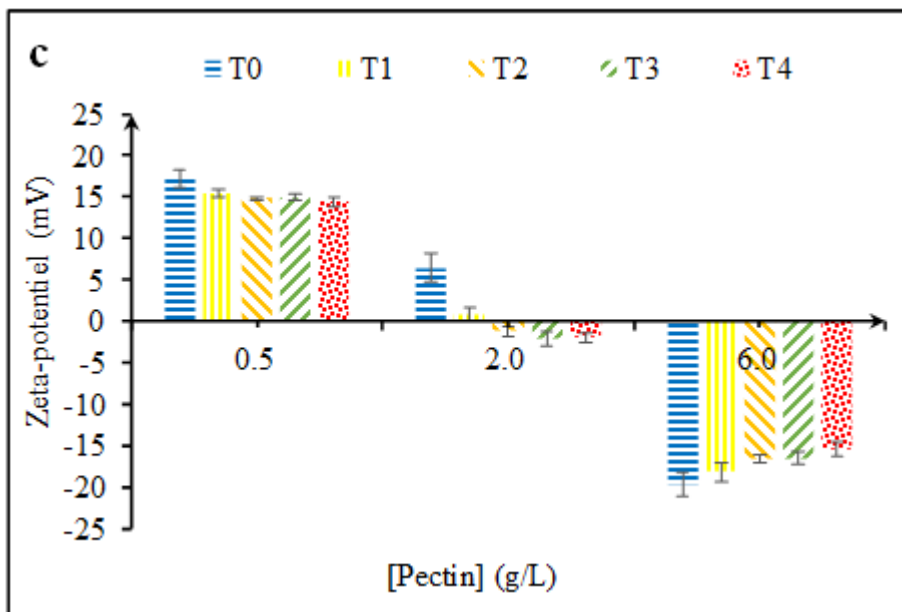
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368 **Fig. 5**



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