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Electrostatic complexes of whey protein and pectin as foaming and emulsifying agents

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1 **ELECTROSTATIC COMPLEXES OF WHEY PROTEIN AND PECTIN AS FOAMING**
2 **AND EMULSIFYING AGENTS**

3

4 **Running Title: Whey Protein-Pectin Electrostatic Complexes**

5

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7

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16

17

18 **ABSTRACT**

19

20 Five types of electrostatic complex (macromolecular complexes, core-shell particles
21 and mixed homogeneous particles) were formed between whey protein (WPC) and
22 pectin. By controlling the thermal treatment, composition and order of mixing it was
23 possible to produce complexes that for the same biopolymer concentration gave
24 differing functional properties. All protein-pectin complexes showed higher foaming
25 ability and stability than native or heated WPC without pectin. Native WPC had higher

26 emulsifying ability than protein-pectin complexes, but exhibited the lowest emulsion
27 stability. Ingredients based on such ideas might offer the food manufacturer greater
28 control over food structure, stability and organoleptic properties.

29

30

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33 **Keywords:** whey protein concentrate; pectin; electrostatic complexes; foaming
34 properties; emulsifying properties.

35

36 INTRODUCTION

37

38 Biopolymer microparticles or complexes have been investigated as functional
39 ingredients in a range of industries mostly the food industries and more recently the
40 pharmaceutical industries as a drug-delivery system [1, 2]. They have also been
41 shown to be useful in the delivery and protection of bioactive compounds in foods [1-
42 3]. They have also been used to replace fats in foods due to their ability to mimic the
43 sensory, optical and rheological properties of lipid droplets [4, 5]. In view of this, there
44 has been a growing interest in the fabrication of biopolymer microparticles made from
45 proteins alone [6, 7] or mixed protein and polysaccharide complexes [3, 8-13].
46 Molecular complexes form via interaction (commonly electrostatic) between two
47 biopolymer molecules. Particle aggregates form through, for example, heat-induced
48 aggregation of biopolymers. Particle aggregates can be homogeneous, or
49 heterogeneous. Homogeneous particles are characterised by an even distribution of
50 one or more biopolymer types throughout the particle. Heterogeneous particles may
51 consist of a second phase dispersed in a biopolymer particle, or droplets such as lipid
52 emulsion droplets dispersed in the biopolymer particle. Heterogeneous particles may
53 also have a core-shell structure where one biopolymer forms a particle aggregate
54 (core) and a second forms a layer (shell) on the surface. Other particle types that can
55 form are fibrils or non-spherical particles [3].

56

57 Proteins and polysaccharide form electrostatic complexes when they have
58 opposite electrical charge [3, 9, 14]. For example, the electrical charge on pectin and
59 whey protein molecules is negative at all pH for pectin and at pH above the isoelectric
60 point of the whey protein. At pH below the protein isoelectric point, the net electrical

61 charge changes to positive. Polysaccharide molecules that contain acidic groups are
62 negatively charged over a wide pH range. It is possible to identify a pH range over
63 which the whey protein is positively charged and the pectin negatively charged and
64 thus can form electrostatic complexes. At pH above the protein isoelectric point where
65 the protein is negatively charged, protein and pectin do not interact strongly as a result
66 of electrostatic repulsion between the molecules. When the pH is reduced complex
67 formation will occur at pH's below the isoelectric point. The complexes are soluble if
68 the charge on the protein is not too high, but if the pH is reduced to far below the
69 isoelectric point, extensive complex formation occurs and this eventually leads to
70 precipitation [9, 15, 16]. The complex formation also depends on other factors such as
71 temperature, ionic strength, protein:pectin ratio and protein concentration [3, 17, 18].

72

73 Due to the gelation and aggregation characteristic of protein/polysaccharide
74 complexes, they can influence the colloidal stability and textural characteristic of food
75 products [8, 19-21]. This has found application in a range of new food formulations
76 [22]. Under some conditions proteins and polysaccharides, including whey protein and
77 pectin, undergo phase separation when mixed due to thermodynamic incompatibility,
78 arising from a positive free energy change on mixing [19]. This usually occurs under
79 conditions where the proteins are uncharged or have the same charge as the
80 polysaccharide.

81

82 According to Bouaouina et al. [23], the functional properties of food proteins in
83 combination with polysaccharides, can be categorised into three classes: (a) gelation
84 and aggregation properties, (b) hydration properties (which includes wettability,
85 swelling, dispersibility, viscosity, water holding capacity, adhesion) and (c) interfacial

86 properties, which includes foaming and emulsification properties.
87 In this study we use different fabrication techniques to make whey protein concentrate-
88 high methoxy pectin complexes under conditions where they interact by electrostatic
89 means. The effect that fabrication method has on particle structure, foaming and
90 emulsifying properties is studied to determine the potential of these particles as
91 functional food ingredients.

92

93 **MATERIALS AND METHODS**

94

95 **MATERIALS**

96

97 Whey protein concentrate (WPC) (Lacprodan 87) was kindly donated by Arla
98 Foods Ingredients, Arhus, Denmark. The protein concentration as reported by the
99 manufacturer was 87%. Citrus peel pectin (GENU[®] High Methoxy Pectin ISO USA-
100 SAG type B rapid set 72% DE) was also kindly donated by CP Kelco, Denmark. All
101 sample suspensions were prepared using milli-Q water. Sodium hydroxide (solid
102 pellets) and hydrochloric acid solutions (32% by volume) used for adjusting the pH
103 were purchased from Sigma Aldrich (UK) and Fisher Scientific (UK) respectively.

104

105 **BIOPOLYMER SUSPENSION PREPARATION AND FABRICATION TECHNIQUES**

106

107 WPC and pectin complex (WPP) sample suspensions were made by
108 dissolving the appropriate mass of powder in the appropriate mass of water to give a
109 known concentration of WPC or pectin solution (w/w). The solutions were stirred
110 overnight at room temperature using a magnetic stirrer to ensure they were dissolved

111 and hydrated. To remove insoluble material both protein and pectin suspensions were
112 centrifuged at 3000 rpm for 15 mins (Denley BS400 centrifuge, England) and the
113 supernatant removed. The pH of the solutions was adjusted to pH 4 using 1M HCl
114 solution. pH 4 was chosen because the electrostatic interaction between pectin and
115 whey protein concentration (WPC) is strongest when the pH of WPC is slightly below
116 its isoelectric point ($pI \approx 4.3$) [3, 9, 14]. Samples were left standing at room temperature
117 for 20mins with continuous stirring at the desired pH before further use [3, 11, 12].

118

119 To form molecular complexes or protein-polysaccharide particles, protein and
120 pectin solutions were mixed in the appropriate mass ratio and then heated together or
121 heated individually before mixing with the other (see Table 1). After the samples had
122 been heated in the water bath, they were removed and placed immediately into an ice
123 bath for 3 hours to ensure rapid cooling and to bring all aggregation reactions to a halt.
124 The pH of each solution was checked and readjusted to pH 4 if necessary. The
125 protein-pectin complexes were formed by mixing protein and pectin solutions in the
126 appropriate mass ratio. The samples were then kept in a refrigerator (5°C) overnight
127 prior to analysis. A sample where neither WPC nor pectin were heated, but were still
128 mixed in the appropriate mass ratio was also made and treated in the same way as
129 the heated samples. Table 1 summarizes the fabrication method for each sample.

130

131 To determine conditions for the fabrication of WPC pectin complexes preliminary
132 experiments were carried out to determine the effect of heating temperature, heating
133 time, and protein:pectin ratio on the size of WPP particles. Based on these experiments
134 (Supplementary material Figures S1-S3) a heating temperature and time combination
135 of 80 °C and 25 mins and a protein:pectin ratio of 5:1 was chosen for the formation of

136 WPP particles. The temperature of 80 °C was chosen as this is known to be close to
137 the temperature at which β -lactoglobulin, the major whey protein, starts to denature. In
138 addition 80 °C was the temperature at which many of the particles have the largest size
139 (supplementary material figure s1). The heating time of 25 mins was chosen because
140 all aggregation reactions are complete by this time (Supplementary material Figure S2).
141 Finally, a protein:pectin ratio of 5:1 was chosen as this gives the biggest differentiation
142 in size between the particles, and was stable for the two molecular complexes, which
143 showed very large particle sizes at lower protein:pectin ratios below 4:1
144 (Supplementary material Figure S3). Under these conditions and when mixed and
145 adjusted to pH 4, the pectin and WPC proteins form electrostatic complexes.

146

147 Five types of whey protein-pectin (WPP) particles were fabricated by the
148 manipulation of heat treatment given to each sample as listed in Table 1. It has been
149 shown by other researchers that biopolymer particles can form at a pH just below
150 the isoelectric point (pI) of the protein [10]. Under these conditions WPC proteins
151 have a net positive charge whilst pectin is negatively charged and complexes are
152 formed through electrostatic attraction. Zetasizer measurements showed that the WPC
153 has a pI \approx 4.5. Therefore since our protein and pectin solutions are at pH 4 we would
154 expect the WPC and pectin to form an electrostatic complex when mixed.

155

156 **PARTICLE SIZE DETERMINATION**

157

158 The particle size of the biopolymer particles were measured using dynamic light
159 scattering with a Zetasizer NanoS (model ZEN1600, Malvern Instruments Ltd, UK).
160 The particle size was measured in backscatter mode using a scattering angle of 173°

161 through a 2mL cuvette of 1.0cm path length filled with 1 mL of sample dispersions. The
162 samples were characterized by the particle size distribution (diameter, nm). This was
163 calculated by the Zetasizer computer software from the intensity of the scattered light
164 using cumulants analysis of dynamic scattering data. WPP and WPC dispersions were
165 diluted with milli-Q water at a ratio of 1:100 (v/v). A solution of this concentration was
166 found to be sufficiently dilute to remove the effects of multiple scattering. All
167 measurement were performed at 25°C and replicated three times [3, 9, 11, 14].

168

169 **DETERMINATION OF FOAMING PROPERTIES**

170

171 The foaming property of WPP complex suspensions were determined by the
172 method described by Philips *et al.* [24]. Foams were formed by whipping 300mL of
173 sample dispersion (1.65 wt% WPC and 0.33 wt% pectin) in a 3 litre capacity double
174 beater mixer bowl (Breville SHM1, COMET, UK) at room temperature. Each sample
175 was whipped for 5 mins whilst the rotational speed was set at 5 (maximum). The
176 foaming ability and foam stability were then measured by the methods described
177 below.

178

179 **FOAMING ABILITY**

180

181 The foaming ability was estimated by measuring the volume (mL) of foam
182 formed by each sample immediately after whipping was stopped. The method was
183 simple and suitable for the purpose, and it was designed in our laboratory to serve the
184 purpose. To enable estimation of foam volume a standard curve of volume of sample
185 vs measured height of sample in the bowl was plotted. This was carried out by filling up

186 the whipping bowl with a varied volume of sample suspension and then measuring the
187 height of fluid. The height of suspension and the corresponding volume was then used
188 to plot a standard curve (data not shown) which was fitted to a linear equation (Equation
189 1). Foam volume was then calculated by measuring the height of foam in the bowl and
190 using equation 1 to calculate the volume.

191

$$192 \text{ Foam volume} = (297.6 \times \text{height of foam}) - 160.1 \quad (1)$$

193

194 **FOAM STABILITY**

195

196 The foam stability was determined at room temperature by the drainage method
197 described by Phillips *et al.* [24]. To ensure continuous measurement of liquid drainage
198 from foams, a 0.6 cm hole was drilled 5.0 cm from the centre of the bottom of the
199 whipping bowl. The hole was sealed during whipping with a rubber stopper. After 5
200 mins of whipping, the rubber stopper was removed and the bowl was seated on a
201 glass funnel placed in a ring stand at a 30° angle above a measuring cylinder so that
202 the drainage hole was at the lowest point. The drained liquid was collected into the
203 measuring cylinder and the cumulative increase in liquid volume was continuously
204 measured and recorded. The time taken for the foam to drain 50% (i.e. 150 mL) of its
205 initial volume (i.e. before whipping) was used as a measure of foam stability (i.e. the
206 half-life of the foam).

207

208 **PREPARATION OF EMULSIONS**

209

210 Oil-in-water emulsions containing 30% sunflower oil (w/w), 6% (w/w) whey

211 protein-pectin complex (WPP, ratio 5% WPC: 1% pectin) were prepared. Sunflower
212 oil was purchased from Tesco Supermarkets (Edinburgh, United Kingdom). The
213 emulsions were made by premixing the oil and WPP solution using an Ultra Turrex
214 high shear mixer, followed by high pressure homogenization with an APV systems
215 homogeniser (Model APV 1000, Albertslund, Denmark) using a single stage
216 homogenization at 200 bar pressure with continuous recirculation for 15 mins [14, 20].

217

218 **EMULSIFYING ABILITY**

219

220 The emulsifying ability was determined by measuring the oil droplet size in an
221 emulsion made with the WPC and WPP samples. Oil droplet size ($d_{4,3}$) and the droplet
222 size distribution were measured using a Malvern Mastersizer 2000 (Malvern
223 Instruments, Worcestershire, UK) within one minute of making the emulsion. The
224 particle refractive index, solvent (water) refractive index and the assumed absorbance
225 were 1.47, 1.33 and 0 respectively. The $d_{4,3}$ was chosen to express the average
226 particle size because this is more sensitive to any small populations of large droplets
227 that may be formed during homogenization [14, 20].

228

229 **EMULSION STABILITY**

230

231 The emulsion stability of each sample was observed over a 24 week (six
232 months) period by following changes in the particle size distribution and serum
233 separation due to creaming. Sodium azide (Sigma Aldrich, UK) was added to each
234 emulsion sample to prevent microbial growth or spoilage during the storage period.

235

236 For particle size analysis the emulsions were left to stand in transparent plastic
237 bottles at room temperature (25°C) for 24 weeks. The droplet size ($d_{4,3}$) and particle
238 size distribution was measured using a Malvern Mastersizer at time = zero (the day
239 samples were made and within 30 mins of manufacture), and after 1, 2, 3, 5, 10, 18
240 and 24 weeks of storage. For measurement of creaming stability the emulsion samples
241 were stored in 15mL plastic tubes (with added sodium azide) at 25°C for 24 weeks
242 and the serum fraction that separated over the storage period was measured (in mm)
243 at various times over this period. The change in $d_{4,3}$ and cream height were found to
244 be approximately linear over time and the slope of the plots of $d_{4,3}$ or cream height vs
245 time were used as an indicator of the rate of emulsion instability.

246

247 **STATISTICAL ANALYSIS**

248

249 Where statistical analysis was carried out, SPSS (version 22.0) was used to do
250 a one-way analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) to
251 establish significant differences ($p < 0.05$) between results. All tests were replicated
252 three times.

253

254 **RESULTS AND DISCUSSION**

255

256 **PARTICLE SIZE**

257 Based on the methods used to make the WPP particles and the descriptions of
258 electrostatic complex particle structure by Jones & McClements [3] we can
259 hypothesize about the structure of the WPP particles in this study. Heating of WPC
260 leads to denaturation and aggregation [25] whilst prolonged heating of pectin is known

261 to lead to depolymerisation [26-28]. Since in WPP01 the protein and pectin are mixed
262 and heated together we expect these to gel and to form a homogeneous spherical
263 particle. We envisage that the WPP01 particle has a composite mixed structure of
264 aggregated WPC intermixed with degraded pectin. Matalanis et al. [29] have described
265 such a structure as a "heterogeneous continuous" biopolymer complex and are similar
266 to the "type 2" particles described in the work of Jones et al. [12]. For WPP02 and
267 WPP03 the protein is heated first and then mixed with pectin after heating. The pectin
268 will form a layer on the surface of the protein particles through electrostatic interaction
269 to form heterogeneous core shell particles with a core of denatured WPC and an outer
270 shell of pectin. In WPP03 only the protein is heated and the WPC aggregates become
271 coated with an extensive layer of oppositely charged pectin molecules giving particles
272 larger than that formed by WPC alone (Figure 1 & Figure 2). In WPP02, WPC and high
273 methoxy pectin are unstable under heating conditions. When heated the individual
274 proteins in WPC denature and aggregate to form protein particle, and this becomes
275 coated with degraded pectin molecules that are smaller than the unheated pectin and
276 so WPP02 particles are smaller than those of WPP03 (Figure 1 & Figure 2). Various
277 authors [3, 29, 30] have described a similar structure as a "heterogeneous core-shell"
278 biopolymer complex that are the same as the "type 1" particles described by Jones et
279 al. [12]. With WPP04 and WPP05 the protein is unheated, and will form a
280 macromolecular complex with the unheated (WPP04) or heated pectin (WPP05). For
281 WPP04 and WPP05, protein molecules (and pectin (WPP04)) were not heated so
282 extensive aggregations of protein will not occur. However, we hypothesize that the
283 aggregation mechanism in these samples can be explained in terms of complex
284 coacervation. Various researchers [9, 16, 31, 32] describe complex coacervation as a
285 spontaneous separation of a biopolymer system in which one phase is rich in the two

286 biopolymers and the other is depleted of the two biopolymers. It is likely that the
287 aggregation mechanism of the two samples was similar except for the fact that in
288 WPP05 the pectin was heated and this would cause structural change in the pectin,
289 whilst WPP04 was unheated. In both WPP04 and WPP05 we would expect the
290 oppositely charged protein and pectin to associate through electrostatic interactions,
291 with smaller individual protein molecules attached to the larger pectin chains. WPP04
292 and WPP05 particles are of a similar size which may indicate that they are formed
293 through interaction of several protein and pectin molecules, which may be held
294 together through the WPC molecules acting as a “bridge” between the pectin
295 molecules. The heated WPC will also form spherical particles, but these will be
296 homogeneous as they are only composed of WPC. The WPC and WPP particles form
297 a range of particle sizes which can be described by a particles size distribution. The
298 distribution of particle size for both native and heated WPC is shown in Figure 1a and
299 1b and indicates a broad range of particles present naturally or formed during heating.
300 For the native unheated WPC (Figure 1a) these aggregates formed as a consequence
301 of the heat applied during processing of the powder [33]. The particle size distributions
302 for WPP01-WPP03 (Figures 1c-1e) reveal that the particles formed under these
303 conditions have a lower degree of polydispersity than the other particles. It also reveals
304 that some WPP01 (Figure 1c) particles were larger than in WPP02 (Figure 1d) and
305 WPP03 (Figure 1e), but the average was reduced by a population of smaller particles.

306

307 **FOAMING PROPERTIES**

308

309 **FOAMING ABILITY**

310 The ability of WPP particles, native and heated WPC to form and stabilise foams

311 was assessed. All WPP samples showed a highly significant increase in foaming ability
312 over the native and heated WPC samples. Within the set of WPP samples, all samples
313 showed significant difference ($p < 0.05$) in foaming ability except for WPP01 and WPP02
314 (Table 2). Table 2 shows that sample WPP04 had the highest foaming ability (2470 mL),
315 followed by sample WPP05 (2307 mL), while the heated WPC had the lowest foaming
316 ability (1160 mL).

317

318 Based on the results above there was no apparent correlation between the WPP
319 particle size and foaming ability. This result was in contrast with the relationship
320 observed between whey protein aggregate size and foaming ability by other researchers
321 [34-36]. For example, Rullier et al. [36] report that foams made with β -lactoglobulin
322 aggregates had a lower foaming ability and foam stability than those made with the
323 native protein. Furthermore, foaming ability and foam stability decreased with increasing
324 aggregates size in contrast with our own results. However, when native protein was also
325 present in the foaming solution as well as protein aggregates, more stable foams were
326 formed than for the native protein alone. To explain this, Rullier et al. [36] believe that
327 the lower surface activity of the protein aggregates means they are not able to form fine
328 air bubbles in foams, and the larger bubbles are less stable. However, if sufficient native
329 protein is present this can form fine foam bubbles which are stabilised more efficiently
330 by the large protein aggregates. Various researchers [37-40] explain that foaming ability
331 is guided by the surface tension and the rate of diffusion of particles onto the air-water
332 interface. Particles that diffuse rapidly to the air-water interface and are able to reduce
333 surface tension rapidly will stabilise the air bubbles in foam more quickly, and will give
334 rise to smaller bubbles and a greater foam volume. So, a possible explanation for the
335 correlation between increasing particle size and decreasing foaming ability is that the

336 surface tension at the air-water interface is lower when smaller particles are adsorbed
337 than for bigger particles (which also diffuse more slowly to the interface). The results of
338 Rullier et al. [36] confirm this as they have measured surface tension for β -lactoglobulin
339 aggregates and have found that the larger the aggregates the slower the rate of
340 decrease of surface tension, and the higher the final equilibrium surface tension. The
341 differences between our foaming ability results for protein-pectin particles, and those
342 observed by others for protein-only aggregates suggests that the composition and
343 structure of the particles plays a more important role in WPP foaming properties than
344 does particle size. The differences observed between complex particles (WPP) and
345 whey protein-only samples (WPC) is related to the presence or absence of pectin, and
346 it is likely that the increased viscosity caused by pectin is a major contributor to foaming
347 ability. The increased viscosity of the aqueous phase in pectin containing foams helps
348 to trap air bubbles and reduce bubble coalescence, thus leading to a smaller average
349 bubble size. However, clearly it is not only the presence or absence of pectin that is
350 important otherwise all pectin containing samples would have the same foaming
351 properties. The state of the pectin and how it interacts with the protein in the WPP
352 aggregates is also important.

353

354 **FOAM STABILITY**

355

356 The foam stability is a measure of the time it takes for the foam bubbles to burst
357 or rupture. The time taken for this to occur depends on the nature of the stabilising
358 particles at the air-water interface. Table 2 shows significant differences in foam
359 stability among samples ($p < 0.05$). WPP samples produced significantly more stable
360 foams than the native and heated WPC. Samples WPP04 and WPP05 formed the most

361 stable foams with half-lives of ≈ 17 mins and ≈ 14 mins respectively (Table 2) whilst
362 heated WPC (WPC-H) has the lowest foam stability with a half-life of 20 seconds. The
363 foam stability also did not show any correlation or dependence on the particle size.
364 The latter is probably a result of the reduced surface activity of the aggregated proteins
365 mentioned in the discussion of foaming ability. Although the aggregated WPP samples
366 (WPP01, WPP02 and WPP03) are likely to have a reduced ability to adsorb at the air-
367 water interface their larger size will allow them to form thicker more dense adsorbed
368 layers at the air bubble interface. The adsorbed layer in these systems provides a
369 greater stability to the bubbles against coalescence.

370

371 The different types of WPP particle exhibited differing foaming ability and foam
372 stability. The key observations in this respect are (i) the presence of pectin improves
373 foaming ability and foam stability; (ii) the molecular complexes (WPP04 and WPP05)
374 showed a statistically significant higher foaming ability and foam stability than other
375 WPP particles; (iii) the homogeneous spherical WPP01 particles had a substantially
376 lower foaming ability than core-shell particles of WPP02, indicating that where the
377 pectin is located within the aggregate structure is highly important to functionality.

378

379 It is not unsurprising that the presence of pectin improves foaming properties
380 as this will increase the aqueous phase viscosity, a factor known to increase foaming
381 ability and foam stability [41]. However, pectin was present in all WPP particles, so
382 this cannot alone explain the differences between the foaming properties of the WPP
383 aggregates. Simply adding pectin (heated or unheated) to native unheated WPC gave
384 macromolecular complexes with the highest foaming ability and foam stability. In these
385 systems it is possible that the complex formed acted in a similar way to some naturally

386 occurring protein-containing polysaccharides such as gum arabic [42, 43]. That is the
387 protein inferred some hydrophobic character on the pectin molecule and allowed it to
388 adsorb to the air-water interface to allow formation of foam bubbles. At the same time,
389 the pectin part of the complex located in the aqueous phase, and increased the
390 viscosity in the foam plateau borders which reduced the rate of foam drainage and
391 increased foam stability.

392

393 We can also compare the state of the pectin in WPP04 and WPP05 to the pectin
394 found in WPP01 and WPP02. In these the pectin was either incorporated into the
395 aggregate with the protein (WPP01) or sat on the surface of the protein aggregate
396 (WPP02). In both cases the pectin was likely to be in a state where it has a reduced
397 interaction with the water phase and consequently a reduced effect on the viscosity.
398 Thus, we might explain the reduced foaming properties of WPP01 and WPP02
399 compared to WPP04 and WPP05 as being due to a reduced effect of the pectin on
400 aqueous phase drainage in the foam. If this interpretation is correct then clearly the
401 state of the pectin in WPP01 is such that its effect on aqueous phase viscosity is less
402 than that of pectin in WPP02 as the foam stability of WPP01 particles is much less
403 than that of WPP02.

404

405 **EMULSIFYING ABILITY**

406

407 The mean particle size for native WPC, heated WPC and WPP particles and
408 mean particle size ($d_{4,3}$) for emulsions made with these are shown in Figures 3 and 4.
409 The largest protein-pectin particles were formed for WPP03, the heterogeneous core
410 shell particle, where we hypothesize that the particle was made up of a core of

411 aggregated protein, with a layer of pectin electrostatically bound to the surface. The
412 smallest particles were found in the unheated WPC solution (Figure 3). The order of
413 increasing WPP particle size was WPC < WPC-H < WPP05 < WPP04 < WPP02 <
414 WPP01 < WPP03. If we compare the relative protein-pectin particle sizes for the
415 emulsion experiments with those used in the foaming experiments, we observe a
416 strong linear correlation (Supplementary material Figure S4). This gives us confidence
417 that the mechanism of formation and the structure of the two sets of particles are the
418 same, albeit with larger particle sizes at the higher protein+pectin concentrations used
419 to make emulsions.

420

421 In Figure 4 the emulsifying ability of the WPC and WPP particles are presented
422 expressed as the $d_{4,3}$ of the emulsion droplets, where a smaller particle size indicates
423 a better emulsifier. The order of increasing emulsifying ability is WPP01 < WPP04 <
424 WPP05 < WPC-H < WPP02 < WPP03 < WPC. If the emulsifying ability is plotted
425 against the size of the WPC-pectin particles no correlation is observed between the
426 two (data not shown). This suggests that the emulsifying ability is independent of the
427 particle size of the emulsifiers, a finding in agreement with those of Ghosh and
428 Bandyopadhyay [22]. However, it is clear that the conformation of the protein
429 molecules within the complex do have an impact on the emulsifying ability of all the
430 samples. The WPC was the best emulsifier, which is perhaps not unexpected. The
431 proteins in WPC powder are largely un-aggregated, will adsorb readily to the oil droplet
432 surface and are accepted as being good at stabilizing the interface. A similar response
433 has been observed in dissociated caseins which were significantly better emulsifiers
434 than large aggregates of proteins, such as micelle fragments of milk caseins found in
435 skim milk powder and milk protein concentrate [44, 45]. This was because the

436 aggregated proteins do not spread as easily at the oil-water interface and are thus less
437 efficient at stabilizing oil droplets. The adsorption of protein at an oil-water interface is
438 followed by the unfolding of the protein, and this unfolding helps in promoting the
439 interactions and reduction of surface tension [46]. The surface denaturation process
440 is not as efficient in aggregated proteins, as their structure is held together by intra-
441 molecular interactions that oppose surface unfolding. This explains why WPC is the
442 best emulsifying sample, but WPC-H which has been heated and aggregated has
443 reduced emulsifying ability. When pectin is present in the WPP particles, however, the
444 emulsifying ability is modified depending on how the pectin interacts with the WPC.

445

446 The poorest emulsifier of the WPP particles was WPP01 where the protein and
447 pectin were heated together and we believe form a homogeneous spherical particle
448 where the protein and pectin are dispersed evenly through the particle. Here, the
449 surface of the particle is likely to be a mixture of protein and pectin, and clearly the
450 presence of the pectin at the surface interferes with the ability of the particles to adsorb
451 and stabilize the droplet surface. WPP02 and WPP03, where the protein was heated
452 separately, and the pectin was added after (either heated pectin, WPP02, or unheated
453 pectin, WPP03) form a different structure where the pectin forms a layer on the surface
454 of aggregated protein particles through electrostatic interaction. These WPP particles
455 were considerably more efficient as emulsifiers than WPP01 and WPC-H, but not as
456 good as WPC. There was also an effect of pectin treatment observed in WPP02 and
457 WPP03. The WPP03 particle, which contained unheated pectin on the surface of the
458 particles, was a significantly better emulsifying agent than WPP02. Heating of the
459 pectin is believed to lead to degradation via either a β -elimination reaction where
460 atoms or groups are lost from adjacent atoms joined by a single (σ) bond, leading to

461 formation of a double (π) bond [28], or through acid hydrolysis if the pH is low. Thus,
462 it is conceivable that WPP02 had smaller pectin fragments at the surface of the
463 aggregated protein core than were found for the WPP03 complexes. It is possible that
464 this affected the hydrophobicity of the surface of WPP particle, possibly through
465 greater coverage of the surface by the smaller pectin fragments, which made the WPP
466 particle less hydrophobic.

467

468 An interesting observation was made when comparing the size of the WPP
469 complexes with the size of the emulsions made from them. For all emulsions, with the
470 exception of WPP02 and WPP03, the emulsion droplets were significantly larger than
471 the WPP particles. The average particle size of samples WPP02 and WPP03 was 6.7
472 and 5.5 μm respectively, whilst the average emulsion droplet sizes were 6.9 and 3.5
473 μm respectively. This suggests that the WPP02 and WPP03 aggregates cannot be
474 the primary emulsifiers/stabilizers for the emulsion, since they would be too large to fit
475 on the droplet interface. A possible explanation for this effect could be that the
476 WPP02 and WPP03 particles, which were made from heated WPC and unheated
477 pectin were unstable under the high shear conditions of the homogenizer and broke
478 up into smaller particles. The presence of smaller WPP aggregate particles would also
479 explain the relatively small droplet size of the emulsions compared to other WPP
480 samples since smaller aggregates might be expected to be better emulsifiers. An
481 alternative explanation could be that the relatively large protein particles in WPP02
482 and WPP03 contributed to the scattering of light when the particle size was measured,
483 and that the average particle size measured for the emulsion droplets made with
484 WPP02 and WPP03 contained a significant contribution from the protein particles
485 themselves.

486 **EMULSION STABILITY**

487

488 The long-term stability of the emulsions made with WPC and WPP was studied
489 over a period of 24 weeks at room temperature (25°C) by following the change in mean
490 particle size ($d_{4,3}$) and height of cream layer formed. These were measured at weekly
491 intervals and the rate of change of $d_{4,3}$ and rate of creaming determined from the slope
492 of plots of these as a function of time. Plots of these two emulsion stability measures
493 are presented in Figures 5 and 6. The most stable emulsions were formed by the
494 WPP02 and WPP03 complexes, for both change in $d_{4,3}$ with time (Figure 5) and
495 creaming stability (Figure 6). These are the two heterogeneous core-shell particles
496 (Table 1). These two WPP particles performed well in terms of foam stability as well
497 (Table 2) but did not give the highest foam stability. If we look at the least stable
498 emulsions, then WPC had the highest rate of change of $d_{4,3}$ followed by WPP04, whilst
499 for creaming WPP04 was the least stable with WPC more stable to creaming. WPC
500 was anomalous when comparing rate of change of $d_{4,3}$ with creaming rate. For all other
501 WPP particles and WPC-H there was a linear correlation between the two measures
502 of emulsion stability, except for WPC emulsions. A correlation plot is shown in the
503 supplementary material (Figure S5). In this plot the emulsions formed from native
504 WPC appear as an outlier point not close to the best fit line. The order of increasing
505 stability for the emulsions made with WPP particles (excluding WPC) was WPP04 >
506 WPP01 > WPC-H > WPP05 > WPP02 > WPP03 for both creaming and change in $d_{4,3}$.

507

508 To explain the differences in emulsifying ability and emulsion stability a number
509 of factors must be considered. The different WPP structures and sizes will play a role,
510 as might the presence of pectin. We have noted that aggregated proteins are known

511 to be poorer emulsifiers than non-aggregated proteins [44]. Euston & Hirst [44] studied
512 the emulsifying properties of aggregated proteins products (milk protein concentrate
513 and skim milk powder) and compared them to native WPC and sodium caseinate. The
514 aggregated protein products produced emulsions with significantly larger emulsion
515 droplet than the non-aggregated proteins. At low protein emulsifier concentrations, the
516 aggregated protein emulsions were also significantly less stable than those made with
517 the non-aggregated proteins. However, as the protein concentration in the emulsions
518 was increased, a point was reached where the stability of the aggregated protein
519 emulsions increased rapidly, and became greater than that of the non-aggregated
520 proteins. The poor emulsifying properties of the aggregated proteins can be explained
521 by their rigid, compact structure. The aggregated proteins are unable to unfold and
522 spread at the oil droplet surface, and therefore are unable to stabilize the droplets
523 when they are small in the homogenizer. However, the aggregated proteins pack more
524 densely at the oil-water interface, and once the adsorbed layer reaches a certain
525 thickness, Euston & Hirst [44] speculated that the effective density of the emulsion
526 droplets (oil + protein layer) becomes great enough so that the density of the droplets
527 becomes closer to that of the aqueous phase, and creaming reduces. We might expect
528 a similar effect to be observed for our WPP complex particles, since they contained
529 aggregated protein and polysaccharide. The particle size of the aggregates increased
530 in the order WPC > WPP05 > WPP04 > WPP02 > WPP01 > WPP03 and we might
531 expect creaming stability to decrease in this order based on the observations of Euston
532 & Hirst [44]. However, plotting creaming stability (and change in $d_{4,3}$ with time)
533 (Supplementary Figures S6 and S7) against WPP particle size, we find that the
534 emulsion stability characteristics of WPP particles were more complex. There was
535 some indication of a correlation between particle size and emulsion stability, with in

536 general larger WPP particle size leading to more stable emulsions. However, there
537 were two exceptions to this, WPP01 and WPP04, where in both cases the emulsions
538 were less stable than other emulsions containing WPP particles of a similar size. The
539 reason for this lower stability was unclear. WPP01 was a homogeneous particle made
540 when both WPC and pectin were heated together, and were both incorporated into the
541 particle. WPP04 on the other hand was a macromolecular complex formed when
542 unheated WPC and pectin were mixed. With WPP04 it is possible that depletion
543 flocculation was occurring due to the presence of the native pectin molecules.
544 Depletion flocculation is a phenomenon during which the large pectin molecules are
545 eliminated or excluded from the gap between two approaching droplets [47]. This
546 leads to an osmotic imbalance and a net force pushing the emulsion droplets together.
547 Depletion flocculation in emulsions has been observed in the presence of un-adsorbed
548 aggregated caseins [44, 48] and with polysaccharides. This might explain why WPP05
549 emulsions were more stable than those made with WPP04, since the pectin in those
550 complexes had been heated and was likely to be degraded, and thus smaller in
551 molecular weight/size.

552

553 For WWP03, where unheated pectin was also present we did not see a lower
554 than anticipated emulsion stability because we expect the pectin not to be free in
555 solution, but to be adsorbed to the surface of the heated WPC particles to form a core-
556 shell WPP particle. An argument against the depletion flocculation explanation for the
557 low stability of WPP04 emulsions is that depletion flocculation is reversible, and flocs
558 would be expected to dissociate into individual droplets when dispersed in water
559 during particle size analysis with the Mastersizer. However, clearly the Mastersizer
560 detected large droplets in the emulsions. In addition, a depletion flocculation

561 explanation for the low stability of WPP01 emulsions is also not compelling, as there
562 is no clear reason why WPP01 particle would cause this and other particles of a similar
563 size would not. An alternative explanation for the lower than expected stability of
564 WPP01 emulsions is that we could be seeing the effects of a bridging type of
565 flocculation. In emulsions where the protein emulsifier is present in too low a
566 concentration to fully saturate the surface of the oil droplets, the protein can be shared
567 between separate droplets to form a bridge that leads to a more permanent form of
568 flocculation. These flocs are less stable to creaming and to coalescence than
569 individual droplets. The fact that this occurred with WPP01 aggregates and no other
570 aggregates may be because the surface of WPP01 particles might reasonably be
571 expected to be more hydrophobic because it was not fully covered by a layer of
572 hydrophilic pectin as would be expected in a core-shell particle (WPP02 & WPP03).

573

574 **CONCLUSION**

575

576 WPC-pectin electrostatic complexes can be made to adopt, spherical
577 homogeneous particles, core shell particles or macromolecular complexes depending
578 on how the protein and pectin are heated and mixed. This study provides evidence
579 that the structure of WPC-pectin electrostatic complexes can be manipulated to alter
580 foaming and emulsifying properties of their solutions, and suggests that the structure
581 of these particles can be tuned to give optimal foaming and emulsifying properties in
582 in food system applications. It is also possible that similar effects will be observed with
583 thickening and gelation properties and work continues in this area. Our own work on
584 controlling structure in WPC aggregates [6, 7] has shown that control over solution
585 viscosity can be achieved through controlled thermal aggregation so we are hopeful

586 that similar effects can be achieved in the more complex binary aggregates of WPC
587 and pectin. If this is achieved it will open up the possibility of functional ingredients
588 that can be more closely tailored to the functional requirements of a particular product
589 or manufacturer.

590

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592

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595

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734

735 **Table Legends**

736 **Table 1** - Sample fabrication techniques. All heated samples were heated for 25 mins
737 at 80°C (unless otherwise stated).

738

739 **Table 2** - The foaming ability and foam stability of whey protein and protein-pectin
740 complexes. Values are means \pm standard deviation. Means in the same column not
741 followed by the same superscript are significantly different ($p < 0.05$). The methods for
742 manufacture of the WPP particles are given in Table 1.

743

744 **Figure Legends**

745 **Figure 1** - Particle size distribution of whey protein-pectin complexes (WPP) used in
746 the foaming experiments measured by the Malvern Zetasizer. Whey protein-pectin
747 samples have a protein concentration of 0.33% (w/w) pectin and 1.65% (w/w) WPC
748 (protein-pectin ratio of 5:1). The concentration of WPC only samples is 1.65 wt% and
749 pectin is 0.33wt%. WPP samples are prepared according to the methods given in
750 Table 1.

751

752 **Figure 2** – Z-average particle size (μm) of WPP particles used in foaming experiments
753 as measured with the Malvern Zetasizer. Whey protein-pectin samples have a protein
754 concentration of 0.33% (w/w) pectin and 1.65% (w/w) WPC (protein-pectin ratio of
755 5:1). The concentration of WPC only samples is 1.65 wt%. WPP samples are prepared
756 according to the methods given in Table 1.

757

758 **Figure 3** - Z-average particle size (μm) of WPP particles used in emulsifying
759 experiments as measured with the Malvern Zetasizer. Whey protein-pectin samples
760 have a concentration of 1.0% (w/w) pectin and 5.0% (w/w) WPC (protein-pectin ratio of
761 5:1). The concentration of WPC only samples is 5.0 wt%. WPP samples are prepared
762 according to the methods given in Table 1.

763

764 **Figure 4** - Mean particle size $d_{4,3}$ (μm) of emulsion droplets made using WPP particles.

765

766 **Figure 5** – Stability of emulsions made with WPP particles measured as the rate of
767 change of droplet size ($d_{4,3}$ in $\mu\text{m}/\text{week}$).

768 **Figure 6** – Stability of emulsions made with WPP particles measured as the rate of
769 change of cream layer height (mm/week).

770

771 **Table 1**

772

Sample Name	Fabrication technique	Particle Type
WPP01	Heating of mixed WPC and pectin suspensions at pH 4	Homogeneous spherical particle
WPP02	WPC (at pH 5.8) & pectin (pH 7) heated separately and then mixed together and adjusted to a final pH of 4	Heterogeneous core-shell particle
WPP03	WPC (at pH 5.8) heated first, and then mixed together with unheated pectin at room temperature and adjusted to a final pH of 4	Heterogeneous core-shell particle
WPP04	Mixture of unheated WPC & unheated pectin at room temperature adjusted to a final pH of 4	Macromolecular complex
WPP05	Pectin (at pH 7) heated firstly, and then mixed together with unheated WPC at room temperature and adjusted to a final pH of 4	Macromolecular complex
WPC-H	Heated WPC (final pH is 4.0 unless otherwise stated)	Homogeneous particle
WPC	No heat treatment or pH adjustment	Macromolecular solution

773

774

775

Table 2

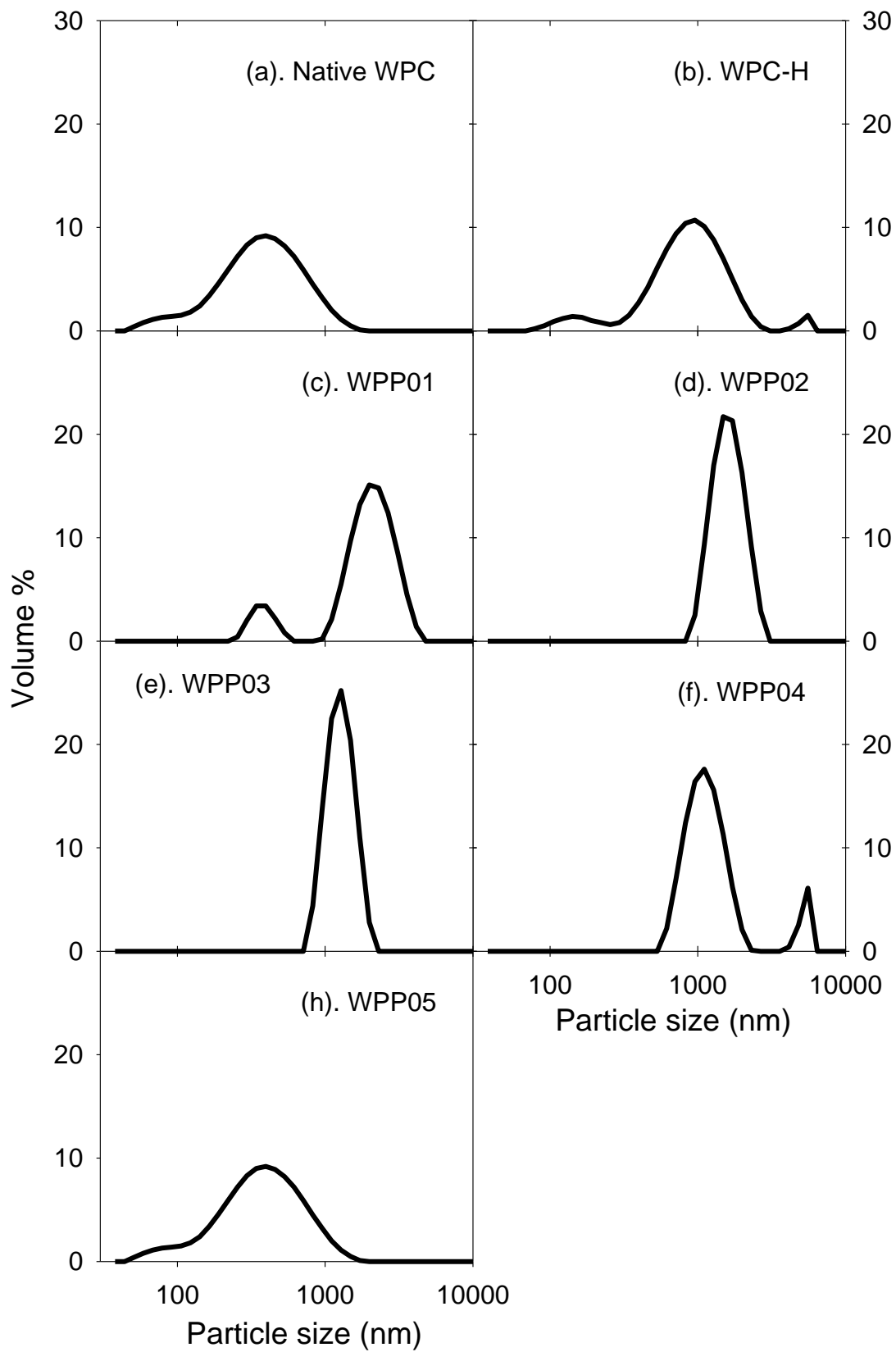
776

Samples	Foaming ability (mL)	Foam stability (sec)
Native WPC	1408±4 ^a	66±0.5 ^a
WPC-H	1160±9 ^b	12±1.1 ^b
WPP01	2233±3 ^c	198±1.4 ^c
WPP02	2247±1 ^c	618±1.9 ^d
WPP03	2030±4 ^d	792±1.3 ^e
WPP04	2470±12 ^e	990±1.7 ^f
WPP05	2307±8 ^f	840±1.0 ^g

777

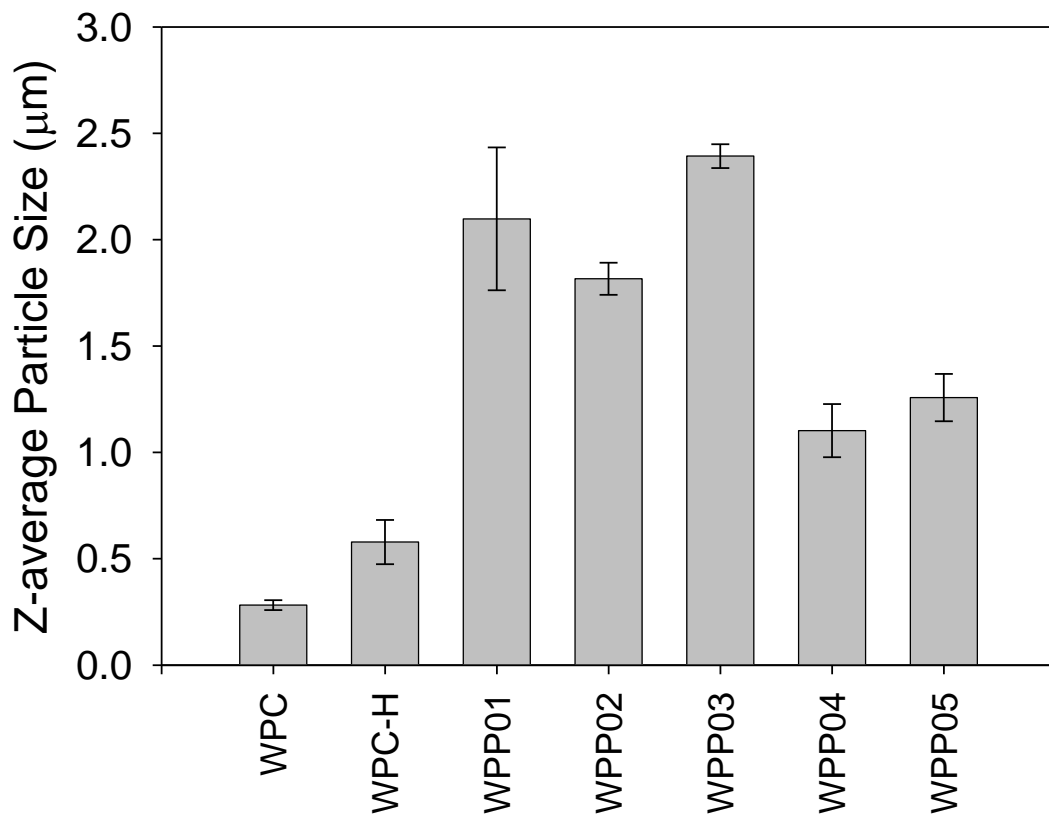
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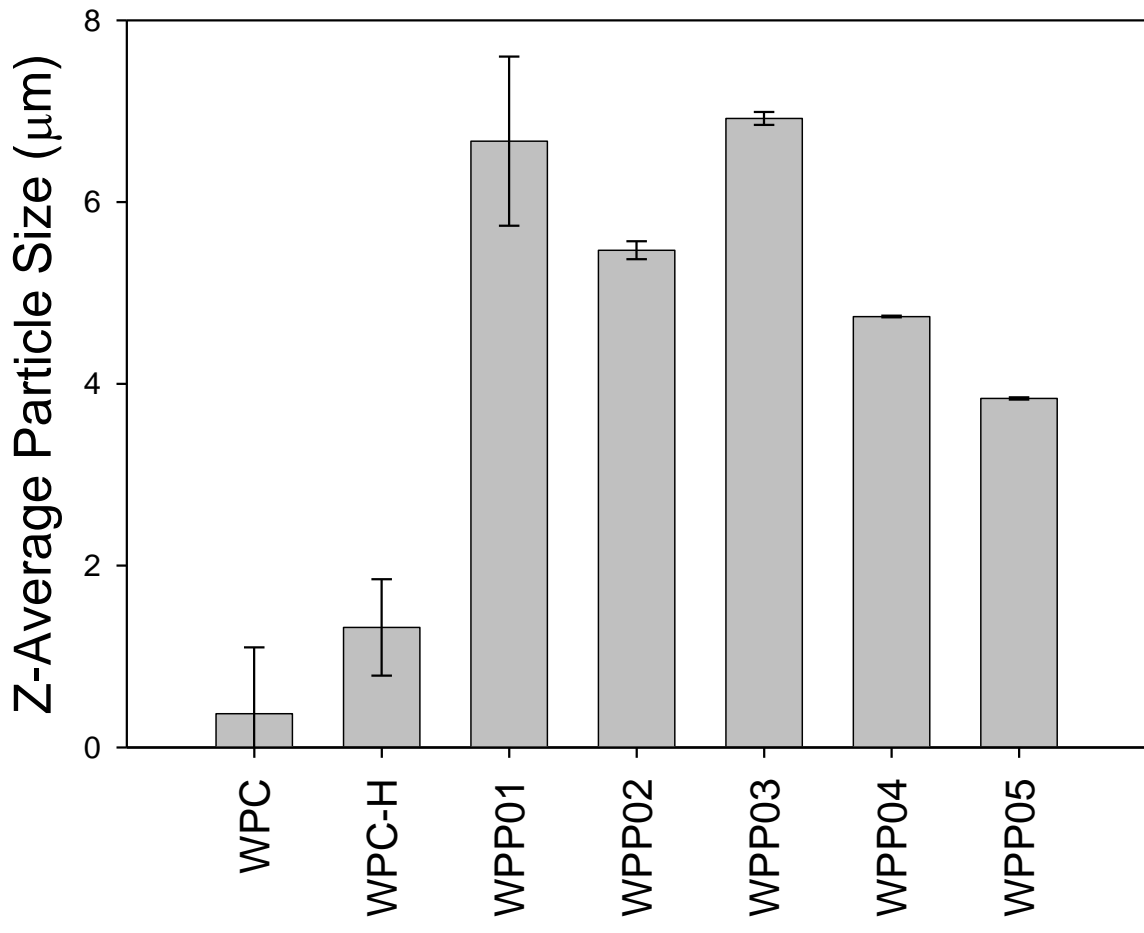
781 Figure 1



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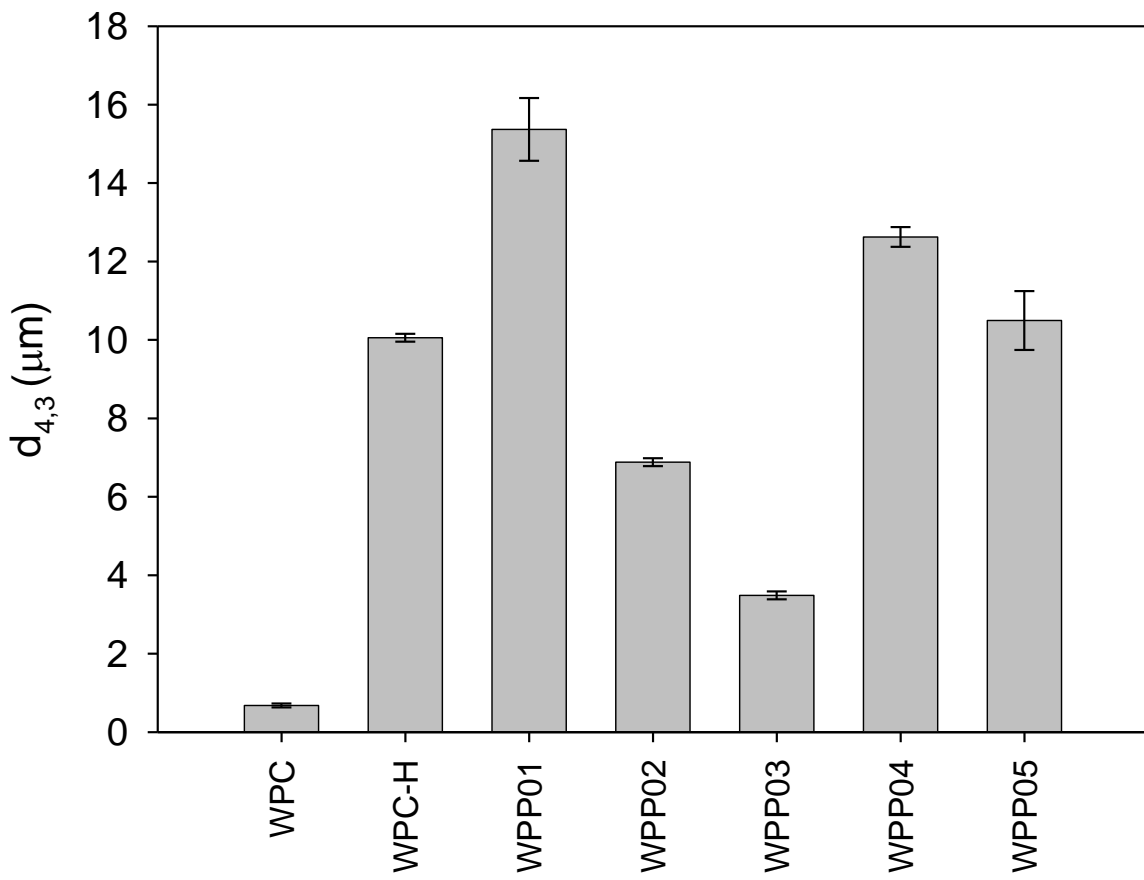
783 **Figure 2**

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Figure 3

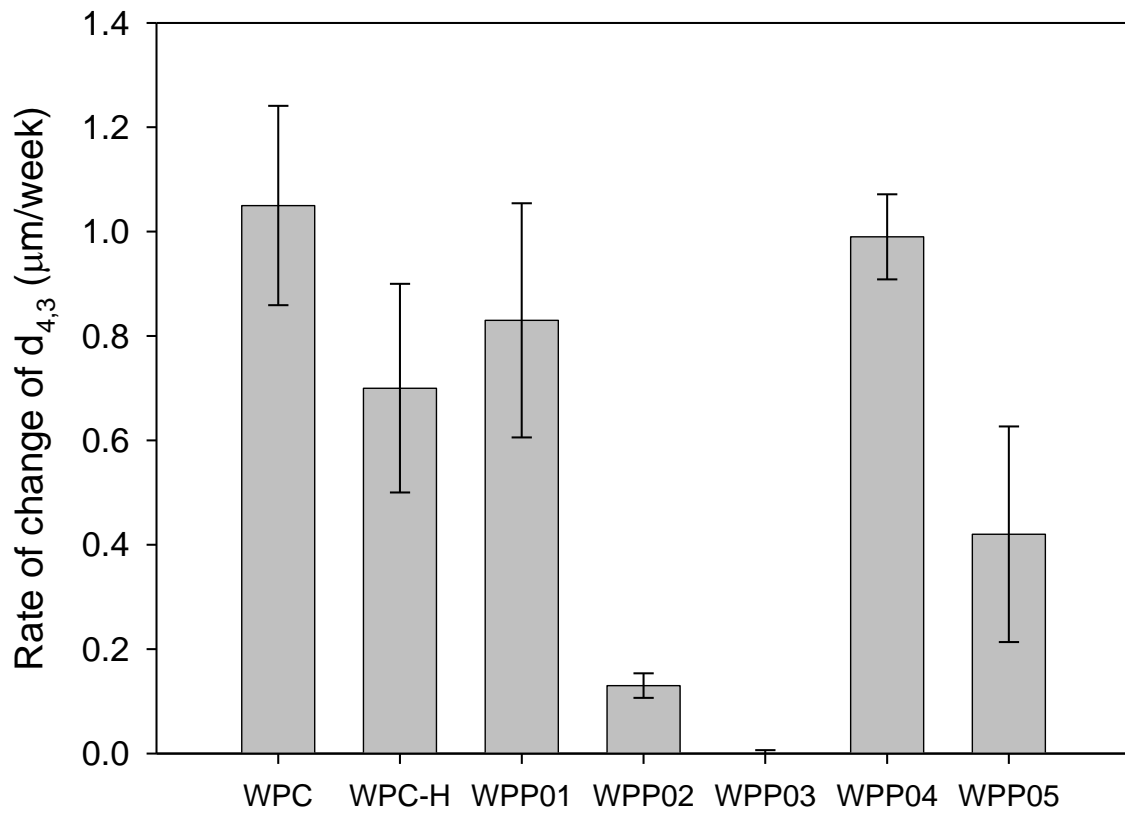


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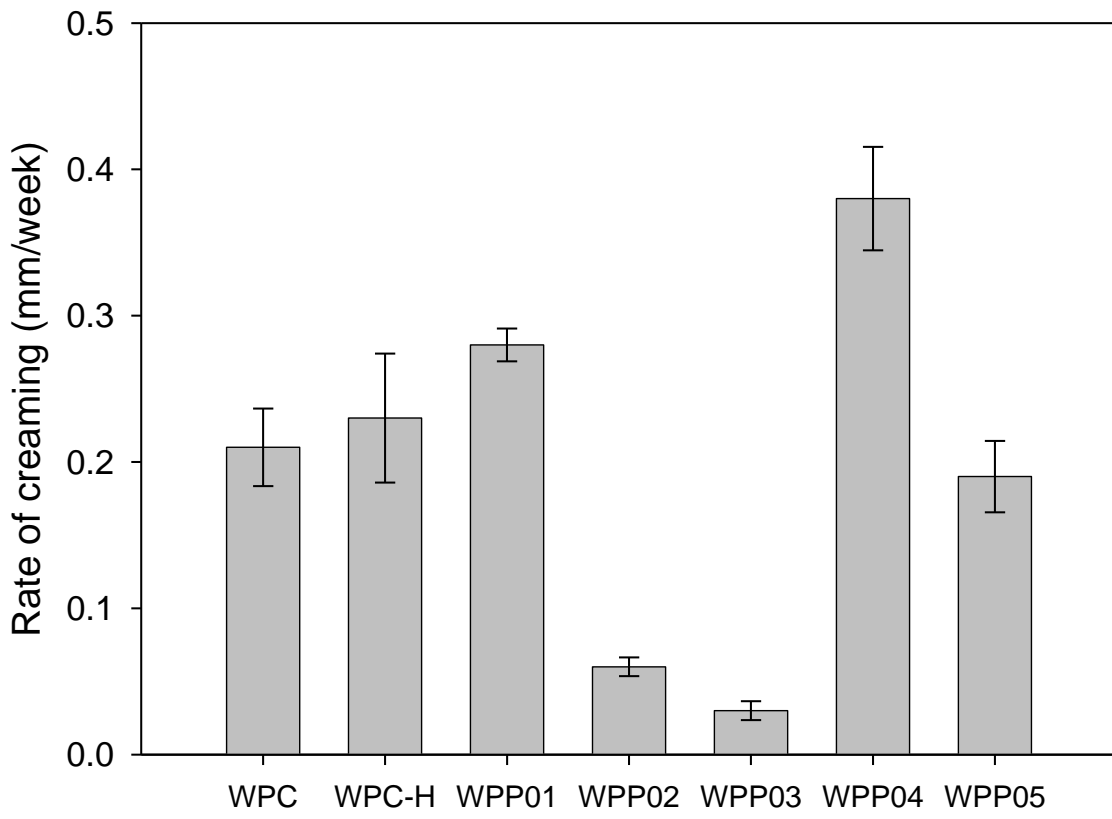
796 **Figure 4**

797



798
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800

Figure 5



801

802 **Figure 6**