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Bioaccessibility of polymethoxyflavones encapsulated in resistant starch particles stabilized Pickering emulsions: Role of fatty acid complexation and heat treatment

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1 **Bioaccessibility of polymethoxyflavones encapsulated in resistant starch particles stabilized**

2 **Pickering emulsions: Role of fatty acid complexation and heat treatment**

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15 **ABSTRACT:** High-amylose maize starch formed complexes with different fatty acids (C12:0,
16 C14:0, C16:0, C18:0 and C18:1) using two hydrothermal methods. The resistances of these
17 starch complexes against enzymatic hydrolysis were all higher than the native starch, while the
18 hydrophobicity of these complexes was enhanced. The capabilities of these starch-fatty acid
19 complexes to form Pickering emulsions were further characterized. Starch-saturated fatty acid
20 complexes were able to form stable emulsions that endured heat treatment of 60, 80 and 100 °C
21 respectively. However, starch-unsaturated fatty acid complexes could not form stable emulsions.
22 The barrier properties of these emulsions were adjusted by swelling of starch granules resulted
23 from heat treatment. Lipolysis profiles of polymethoxyflavones (PMFs) loaded emulsions
24 suggested that certain heat treatment could reduce the accessibility of lipase towards oil droplets
25 and release of PMFs during lipolysis by enhancing the coverage of granules at the oil-water
26 interface. The resistant starch particles stabilized Pickering emulsions have the potential to
27 encapsulate and enhance the bioaccessibility of poorly-soluble phytochemicals in food and
28 pharmaceutical products.

29 **KEYWORDS:** Resistant starch, V-amylose complex, bioaccessibility, fatty acid, Pickering
30 emulsion.

31

1 Introduction

A large variety of compounds with health-promoting biofunctionalities have been recognized in natural plants. However, many of these compounds are lipophilic with poor aqueous solubility, thus possessing low bioavailability over oral ingestion. Encapsulation of bioactive lipophilic compounds using emulsion system has long been applied to increase their solubility and augment their oral dose efficiency. Conventional emulsions formed by small molecular-weight emulsifiers might cause adverse effects, such as irritancy, biological interactions, promoting colitis and metabolic syndrome.[1, 2] An alternative way to avoid use of these surfactants is to apply colloidal particles-stabilized emulsions, so-called Pickering emulsions.[3] Compared to conventional emulsions, Pickering emulsions also possessed higher stability against coalescence and Ostwald ripening.[4] Most of the colloid particles used is from inorganic or synthetic resources.[5-8] Recently, production of particulate emulsifiers from natural biopolymers has gained great attraction, especially with application in food, cosmetic and pharmaceutical industry.[9, 10] Starch is one of the promising materials that has been studied for developing particle stabilizers owing to its overwhelming abundance and inherent biodegradability.[11-13] With intrinsic hydrophilicity, the emulsifying efficiency of most native starch was unsatisfying. Hydrophobic modification of starch is a common approach used to enhance its affinity for oil phase, thus improving its emulsifying properties.[14, 15] Currently, this approach involved the formation of chemical bonding between the hydroxyl groups of starch and the reacting reagents.

The amylose in the starch tends to form single helix structure through complexing with suitable lipophilic molecules.[16] Complexation between fatty acids and starch has been proved to reduce enzyme hydrolysis rate of starch, which could form a type of resistant starch.[17, 18] Since high-amylose maize starch has high gelatinization temperature and relatively higher content of

55 resistant starch compared to normal and waxy maize starches. In our previous research, high
56 amylose maize starch-fatty acid complexes with fatty acid chain length of 12-18 carbons have
57 been prepared.[19] The morphological and structural characterization of the resulting complexes
58 have been fully analyzed. Using a relatively low hydrothermal treatment temperature (80 °C),
59 these complexes exhibit a V-type structure and still maintain the original intact granule shape of
60 native starch.[19] Since medium and long chain fatty acids are lipophilic, the hydrophobicity of
61 starch-fatty acid complexes would be enhanced. Hence, the emulsifying efficiency of these
62 complexes would be improved compared to native starch. In this study, complexes formed by
63 starch and different fatty acids will be used to stabilize Pickering emulsions. This is one pioneer
64 study that using resistant starch as colloid particles to form Pickering emulsions. The resulting
65 Pickering emulsions will possess the health benefits that linked with resistant starch, which
66 include lower the postprandial plasma-glucose, intervene insulin resistance, serve as a potential
67 probiotic and promote colonic health.[20-23] Although starch granules stabilized Pickering
68 emulsions are stable against coalescence, there could be some relatively large space between
69 starch granules at the oil-water interface even if a fully coverage interface was formed due to
70 relatively large size of starch granules. The gelatinization of starch granules at the oil-water
71 interface under heat treatment could adjust the barrier properties of starch granule stabilized
72 Pickering emulsions.[24] Since the formation of amylose-lipid complex in starch granules restricts
73 the swelling of starch granules during heating process and enhance the resistant of starch
74 molecules to enzymatic hydrolysis,[25, 26] the delayed or controlled lipid digestion and controlled
75 release of encapsulated bioactive compounds within the gastro-intestinal tract could be achieved
76 by applying different extent of heating treatment on the starch-fatty acid complex stabilized
77 Pickering emulsions.

78 Polymethoxyflavones (PMFs) are a unique class of flavonoids that exist almost exclusively
79 in the peels of citrus fruits. The health-promoting properties of PMFs are well-documented,
80 which include neuroprotective, anti-inflammatory, antiatherogenic, antiangiogenic and anticancer
81 activities.[27-30] Monodemethylated PMFs are a unique subclass of PMFs, that have been isolated
82 from aged citrus peels and proved to exhibit stronger bioactivities in different systems than their
83 permethoxylated PMF counterparts.[31-34] As lipophilic bioactive reagents, monodemethylated
84 PMFs have low bioavailability over oral consumption due to poor aqueous solubility. The control
85 release of PMFs during digestion process could be achieved by applying appropriated delivery
86 system.

87 Therefore, the aim of this research was to investigate the emulsifying efficiency of resistant
88 starch formed by complexing high-amylose maize starch with different fatty acids to stabilize
89 Pickering emulsions, and barrier properties of resistant starch stabilized Pickering emulsions
90 after different heat treatments. To evaluate their encapsulation and delivery properties of
91 lipophilic bioactive compounds, two key polymethoxyflavones (PMFs), 5-demethyltangeretin
92 and 5-demethylnobiletin were incorporated into the emulsion systems. And the release profiles of
93 the PMFs under *in vitro* small intestinal digestion conditions in Pickering emulsions after
94 different extent of heat treatments were investigated.

95 2 Material and methods

96 2.1 Materials

97 High-amylose maize starch (H-VII, ~70% amylose) was obtained from Ingredion (Bridgewater,
98 NJ). The soy bean oil was purchased from a local supermarket. Lauric acid (C12:0), myristic
99 acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1) with at least 99%
100 purity were purchased from Sigma-Aldrich (St. Louis, MO). Sodium taurodeoxycholate (NaTDC)

101 was purchased from CalBiochem (La Jolla, CA). Phosphatidylcholine (PC75 rapeseed lecithin)
102 was a gift from the American Lecithin Co. (Oxford, CT). Pancreatin from porcine pancreas (Cat.
103 No. P7545) and amyloglucosidase (Cat. No. A7095, activity 300 unit/mL) were purchased from
104 Sigma-Aldrich (St. Louis, MO). 5-Demethyltangeretin and 5-demethylnobiletin at a ratio of 3:1
105 (w/w) with a purity of >98% was synthesized in our laboratory using a previously published
106 method.[35] All other chemicals used in the study were analytical grade.

107 **2.2 Preparation of starch-fatty acid complex resistant starch**

108 The starch-fatty acid resistant starch was prepared according to a previous method with
109 modifications.[19] Two methods were used to prepare the starch-fatty acid complexes.

110 *Heating starch prior to the addition of fatty acid (method I).* Dried native starch mixed with
111 distilled water [40%, w/w, dried starch base (dsb)] in sealed reaction vessels were heated at
112 80 °C for 12 h. Fatty acid (15%, w/w, dsb), dissolved in ethanol (35%, w/w, dsb) was vigorously
113 mixed with heated starch dispersions. The mixtures were uncovered to evaporate the ethanol at
114 80 °C for 5 min, then sealed and further heated for 2 h at 80 °C.

115 *Adding fatty acid prior to heating to the starch (method II).* The fatty acid (15%, w/w, dsb),
116 dissolved in ethanol (35%, w/w, dsb), was vigorously mixed with dried starch. The mixtures
117 were heated at 80 °C for 5 min in reaction vessels without cover to evaporate ethanol, then sealed
118 and further heated at 80 °C for 2 h. Distilled water (40%, w/w, dsb) was added under vigorously
119 stirring and then heated at 80 °C for an addition of 12 h.

120 After the heating process, all the samples from method I and II were cooled to room
121 temperature, washed with 50% distilled water-ethanol solution (v/v) for three times, and
122 recovered by centrifugation (1,500 ×g for 10 min). The resulting pellets were dried at 40 °C
123 overnight and ground to fine powder.

124 **2.3 Lipid content**

125 The lipid contents of the samples were determined according to a previous study.[36] The free
126 lipid in the complexes was measured by extracting using Soxhlet with petroleum ether at 50 °C
127 for 10 h. The total lipid content of the complexes was measured after acid hydrolysis process of
128 starch. Around 1.00 g of complex was accurately weighed and well mixed with 10 ml of distilled
129 water. Then 15 ml of 8.0 M HCl was added before heating in a boiling water bath for 25 min.
130 After the heating process, 50.0 ml of distilled water was added. The mixture was then filtered
131 through filter paper and washed with distilled water until the filtrate reach neutral. The residue
132 with filter paper was dried overnight at 40 °C. The total lipid content of the dried residues was
133 Soxhlet extracted with petroleum ether at 50 °C for 8 h. The recipients with the extracted fat
134 were dried at 105 °C to constant weight. The lipids in the samples were composed by both free
135 lipids and complexed lipids (complexed lipids = total lipids - free lipids). All samples were
136 analyzed in triplicates.

137 **2.4 Contact angle measurement**

138 Starch films were formed using solution casting method. The samples were heated in distilled
139 water at 150 °C. The suspensions were poured onto glass dishes and dried at room temperature
140 overnight. The water contact angle of the films was measured using sessile drop method on a
141 VCA Optima-goniometer system (AST Products, U.S.A). A drop of water (2μL) was placed on
142 the starch film. Then the contact angle was measured at least at five different places of the film.
143 The reported result was the average value of these measurements.

144 **2.5 *In vitro* starch digestibility**

145 *In vitro* digestibility of the samples was analyzed using Englyst method with slight
146 modifications.[21] Starch samples (600 mg, dry base), distilled water (10 ml) and five glass beads

147 were added into 50 ml centrifuge tubes. The tubes were capped and mixed using vortex for 5 min
148 before heated for 30 min in a boiling water bath. The tubes were vortexed in each 5 min intervals
149 to prevent agglomeration during heat treatment. The tubes was then equilibrated in a shaking
150 thermostat at 37 °C for 30 min before 5 ml of sodium acetate buffer containing porcine
151 pancreatin (3×10^3 USP) and amyloglucosidase (40 units) was added. 1.00 ml of aliquots were
152 taken at 20 and 120 min of reaction time, and mixed with 10 ml of ethanol-water solution (80%,
153 v/v) to stop enzyme reaction. The reducing sugar content in the mixture was measured using the
154 3, 5-dinitrosalicylic acid method. Each sample was analyzed in triplicate.

155 **2.6 Preparation of blank emulsions and PMFs loaded-emulsions**

156 Blank emulsions without PMFs was prepared by dispersing starch-fatty acid complexes in
157 distilled water (12 mg/ml water phase) and thoroughly mixing with same volume of soy bean oil
158 using an IKA Ultra-Turrax T25 homogenizer at 12,000 rpm for 3 min. To evaluate the
159 emulsifying ability of different complexes particles, the ratio of complexes particles precipitated
160 at the bottom of the vials (RP) were measured according to equation: RP (%) =
161 $\frac{\text{Mass of precipitated particles}}{\text{Total mass of added particles}} \times 100$. PMFs loaded-emulsions were prepared using the same method
162 as blank emulsions except the oil phase containing dissolved PMFs (2 mg/ml in oil phase), and
163 the oil/water ratio was set up at 5:4. To investigate the effect of heat treatment on the emulsions,
164 the emulsions were heated at 60, 80 and 100 °C for 1 h, respectively. Then samples were
165 equilibrated overnight before further analysis. All samples were prepared in triplicate.

166 **2.7 Microstructure observation of emulsions by light microscopy**

167 The microstructure of starch-fatty acid complexes stabilized Pickering emulsions were
168 analyzed using a Nikon Eclipse TE2000-U (Japan) microscope fitted with a 1392×1040
169 resolution CCD camera (Retiga EXi, QImaging). The emulsions were placed on a glass

170 microscopic plate without cover glass. The mean droplet size of the emulsions was determined
171 by measuring at least fifty emulsion droplets from at least five different parts of the emulsions
172 using ImageJ program.

173 **2.8 *In vitro* lipolysis of PMFs in emulsions**

174 The *in vitro* lipolysis study simulated the digestion conditions of human small intestine and
175 was performed based on a method described previously.[37] Briefly, a fed-state lipolysis buffer
176 with Tris maleate (50 mM), NaCl (150 mM), $CaCl_2$ (5 mM), NaTDC (20 mM),
177 Phosphatidylcholine (5 mM) was prepared. Enzyme suspension was freshly prepared by adding
178 1 g of pancreatin into 5 ml fed-state buffer, stirring for 15 min, centrifuging at 2,000 rpm for 15
179 min, collecting the supernatant, and storing on ice. The lipolysis study was began by mixing
180 Pickering emulsions containing 250 mg oil phase with 9 ml fed-state buffer and keeping at 37 °C
181 for 10 min under stirring. Then the pH of the mixture was adjusted to pH 7.5 before 1 ml of ice-
182 chilled enzyme suspension was added to initiate the digestion. The pH of digestion buffer during
183 2 h digestion process was maintained at 7.5 ± 0.1 by adding 0.25 M NaOH. The volume of added
184 NaOH solution at each time point was recorded throughout the lipolysis experiments. The
185 experiments were conducted in triplicates.

186 The amount of NaOH added was assumed to equal to the amount of free fatty acids (FFAs)
187 released by lipolysis of triacylglycerols. The extent of lipolysis was calculated using the
188 following equation assuming digestion of one triglyceride unit released two molecules of fatty
189 acid and consuming two molecules of NaOH:

190 $$\text{Extent of lipolysis (\%)} = \frac{V_{\text{NaOH}} C_{\text{NaOH}} M_{\text{w,lipid}}}{2\, m_{\text{lipid}}} \times 100 \qquad (1)$$

191 where V_{NaOH} is the volume of NaOH added during lipolysis, C_{NaOH} is the concentration of
192 added NaOH solution (mol per 1000 cm^3), $M_{w,lipid}$ is the average molecular weight of the lipid (g

193 per mol), and m_{lipid} is the total mass of lipid present in the sample (g). The extent of lipolysis at
194 30 min was recorded as a measure of barrier properties of starch-fatty acid stabilized emulsions
195 after different heat treatments on the initial rate of lipolysis.

196 **2.10 Bioaccessibility determination**

197 Upon completion of the lipolysis, the digestion buffers were ultracentrifuged at 4 °C and
198 40,000 rpm for 40 min, which were separated into several phases, an oily phase at the top, an
199 aqueous phase containing incorporated PMFs micelles in the middle, and an opaque sediment
200 phase. The volume of middle phase micelle phase which represented the major forms of
201 compounds for potential intestinal absorption was calculated, and its PMFs concentration was
202 analyzed using HPLC. The bioaccessibility (%) of PMFs was calculated using following
203 equation:

204 $$\text{Bioaccessibility } (\%) = \frac{\text{amount of solubilized PMFs in micelle}}{\text{amount of PMFs in the formulations}} \times 100\% \quad (2)$$

205 **2.11 High-performance liquid chromatography (HPLC) analysis of PMFs**

206 The UltiMate 3000 HPLC system equipped with a 25D UV-VIS absorption detector (Dionex)
207 and Supelco's RP-Amide column, 15 cm × 64.6 mm i.d., 3 μm (Bellefonte, PA) was used to
208 analysis PMFs based on a previous study.[38] The detection wavelength was set at 320 nm. And the
209 injection volume was thirty microliters. The gradient elution with a mobile phase of water (A)
210 and acetonitrile (B) was used. The following elution program was used: 0 to 10 min, linear
211 gradient from 60% A/40% B to 45% A /55% B, then linear gradient to 30% A /70% B at 15 min,
212 followed by linear gradient to 20% A/80% B at 20 min, finally a linear change back to 60% A /40%
213 B at 21 min and lasting for 1 min. The flow rate was set at 1 ml/min.

214 **2.12 Statistical analysis**

215 Analysis of mean values and variances were conducted using Duncan's least significant test (p

216 < 0.05) by SPSS 13.0 statistical software for Windows (SPSS, Inc., Chicago, IL, USA).

217 3 Results and discussion

218 3.1 Lipid content and contact angle of starch-fatty acid complexes

219 When fatty acids and starch were processed under hydrothermal conditions, the fatty acids in
220 the resulting starch-fatty acid complex could exist as free fatty acids and complexed fatty acids.
221 And the complexed fatty acids were mainly from the helical complexation between amylose and
222 fatty acids.[39] The amount of total lipids, complexed lipids and free lipids in starch-fatty acid
223 complexes were analyzed and presented in **Table 1**. The addition mode (before or after heat
224 treatment of starch) and chain length of fatty acids were both found to affect the lipid content in
225 the resulting starch-fatty acid complexes. Generally, the content of free lipids in the starch-fatty
226 acid complexes from both methods I and II increased when the chain length of fatty acids
227 increased from 12 to 18 carbons. Meanwhile, the complexed lipid content of starch-fatty acid
228 complexes decreased with the increase in the fatty acids chain. Complexed lipids might be
229 mainly from the fatty acids that formed single helix complex with linear amylose.[19] The
230 formation of V-amylose complex between amylose and lipids is found to be affected by the fatty
231 acid chain length. Previous studies indicated that the V-amylose formation decreased with
232 increased in the chain length since higher activation energy was needed for complex formation
233 with longer acyl chain.[40] The free lipids could come from the fatty acid crystals that coated on
234 the surface of starch granules or were physically trapped in some regions of starch granules other
235 than within the helixes.[19] Saturated fatty acids with longer aliphatic chain possess higher
236 crystalline temperature and lower solubility in ethanol solutions, which are more susceptible to
237 aggregation and crystallization during hydrothermal treatment. Consequently, increasing the
238 chain length of fatty acids led to higher content of free fatty acid residues in the complexes. The

239 content of free fatty acids and complexed lipids in starch-unsaturated fatty acid complex was
240 much lower than other starch-saturated fatty acid complexes. The decrease of free unsaturated
241 fatty acid content in the complexes could be attributed to the relatively low crystalline
242 temperature and high solubility in ethanol solutions of unsaturated fatty acid. The less efficient
243 complexing between amylose and unsaturated fatty acids might be due to the nonlinear or kinked
244 *cis*-double bound of unsaturated fatty acid requiring a larger helix cavity to accommodate the
245 unsaturated portion of the acyl chain.[41, 42]

246 The addition mode of fatty acids (before or after heat treatment of starch) also impacted the
247 interactions between starch and fatty acids. Starch-fatty acid complexes from method II
248 contained higher amount of total lipids and complexed lipids than their counterparts from
249 method I, while the free lipids content in complexes from method II was lower than that from
250 method I except for starch-lauric acid complex. In method I, addition of fatty acids was after the
251 swelling and partial gelatinization of starch granules, leading to more porous and accessible
252 structure in the amorphous and crystalline lamellae.[43] Therefore, fatty acids were supposed to
253 more easily penetrate into the granules, complex or entangle with the external and internal
254 amylose. Since V-amylose formation was difficult between amylose and fatty acid with longer
255 chain length, a large amount of the penetrated fatty acids might be physically trapped in the
256 amorphous lamellae or between helixes of the crystalline lamellae. These physically trapped free
257 fatty acids were hard to be removed during the washing procedure and retained in the resulting
258 complexes. In the case of method II, fatty acids were added before hydrothermal treatment,
259 which provided longer reaction time between fatty acids and amylose to form V-amylose
260 complex. And the fatty acids might coat on the surface of the starch granules, inhibiting the
261 penetration of other fatty acids into the granules.[44] As a result, most of the free fatty acids were

262 located on the surface of the granules, which could be easily removed by washing solutions. The
263 obtained starch-fatty acid complexes would contain less free fatty acids.

264 The contact angles of these starch-fatty acid complexes were presented in **Table 2**. The
265 contact angles of starch-saturated fatty acid complexes were ranged from 78.6º to 82.6º, which
266 are much higher than that of native starch (46.1º). And the enhancement in the contact angle of
267 the complexes was positively related to their total lipid contents. When the lipid contents of
268 starch-fatty acid complexes prepared by method I increased from 2.84 to 3.47 (g/100 g starch),
269 contact angle of the complexes improved from 78.6º to 81.2º. Then the contact angle declined to
270 78.5º when the lipid content decreased to 3.35 (g/100 g starch). Similarly, the contact angle of
271 complexes from method II decreased from 82.6º to 80.9º with the lipid content decreasing from
272 3.74 to 3.50 (g/100 g starch). Containing higher lipid contents, starch-fatty acid complexes from
273 method II exhibited slightly higher contact angle than their counterparts from method II. But
274 total lipid content wasn't the only factor affecting the contact angle of complexes. Containing
275 same total lipid content of 3.35 (g/100 g starch), samples from Method I-C14:0 and Method I-
276 C18:0 had different contact angles of 79.4º and 78.5º, respectively. This phenomenon could be
277 attributed to the different fatty acids used. Myristic acid and stearic acid were used in samples
278 from Method I-C14:0 and Method I-C18:0. Although, they had the same lipid contents, the
279 amount of fatty acid molecules was higher in starch-myristic acid complex compared to starch-
280 stearic acid complex. Moreover, more complexed lipids and less free lipids presented in sample
281 from Method I-C14:0 than Method I-C18:0. The distributions of fatty acid molecules in the two
282 complexes might also affect the contact angles. Compared to starch-saturated fatty acid
283 complexes, contact angles of starch-unsaturated fatty acid complexes were much lower, which
284 could be attributed to the low lipid content of the complexes. Increasing in the contact angle of

285 starch-fatty acid complexes indicated the improvement of their hydrophobicity, which would
286 adjust their affinity to the oil and water phase, and might enhance their emulsifying efficiency
287 during formation of Pickering emulsions.

288 **3.2 *In vitro* starch digestibility**

289 According to previous classification method,[45] the starch fractions hydrolyzed within 20 min
290 and between 20 and 120 min were referred as "rapidly digested starch" and "slowly digested
291 starch", respectively. And the rest of the starch was categorized as "resistant starch". The
292 contents of RDS, SDS, and RS in different starch-fatty acid complexes were presented in **Table 3**.
293 The RDS, SDS, and RS content of native starch were 75.2%, 6.5% and 18.8%, respectively. The
294 addition mode of fatty acids and chain length of fatty acids were found to affect the enzymatic
295 hydrolysis of starch. After complexing with fatty acids, the SDS and RS content of the
296 complexes were markedly increased. The V-amylose complex formed between amylose and fatty
297 acids is known to be resistant to enzymatic hydrolysis.[46] These V-amylose complexes would
298 restrict the swelling of starch during heating, and the free fatty acids coated on the surface of the
299 starch granules, which could further reduce the accessibility of starch molecules to enzyme
300 digestion.[25, 26] In the case of starch-saturated fatty acids complexes, the content of RDS in the
301 complexes from both method I and II was decreased markedly, and the RS content increased
302 with prolonged fatty acid chain length. The RS level in the starch-fatty acids complexes from
303 method II was slightly higher than that of their counterparts from method I. The higher content of
304 RS in complexes from method II might be result from the more efficient formation of amylose-
305 fatty acid complexes, which could be manifested by the higher complexed lipids content
306 observed earlier. Compared to native starch, the RS content of complexes was increased when
307 complexing with unsaturated fatty acid. However, the RS level was lower than that of starch-

308 saturated fatty acid complexes with same chain length, which might be attributed to lower
309 thermal stability of amylose-unsaturated fatty acid complexes.[42]

310 **3.3 Emulsifying capacity of starch-fatty acid complexes**

311 After complexing with fatty acids, the hydrophobicity of resulting complexes was largely
312 enhanced due to the long aliphatic chain of these fatty acids, which would adjust their affinity to
313 the water and oil phase. The bulk images of Pickering emulsions stabilized by different starch-
314 fatty acid complexes (o/w 1:1, 12 wt%) were presented in **Figure 1**. All the starch-saturated fatty
315 acid complexes were able to stabilize Pickering emulsions, which were creaming quickly after
316 the homogenization due to the large emulsion droplets. The emulsifying volume of emulsion
317 stabilized by starch-lauric acid complex from method I was the smallest, and a noticeable
318 amount of complex was settled down at the bottom. The ratio of precipitated starch-lauric acid
319 complex particle from method I reached 19.7%, which was much higher than that of other
320 complex particles (**Table S1**). These results suggested that its emulsifying ability might be less
321 efficient than other complexes. In the case of starch-unsaturated fatty acid complexes,
322 emulsifying phase seemed to be formed. However, a certain amount of oil phase was leaked out
323 on the top of emulsion phase.

324 The typical microscopic images, combined with average droplet size of these emulsions are
325 presented in **Figure 2**. As evidenced from the images, the mode of adding fatty acids and chain
326 length of fatty acids during complexes processing had a distinct influence on the size of the
327 emulsion droplets. The difference in the emulsifying ability of these complexes was related to
328 wettability of the complex particles at the oil-water interface, which was affected by the total
329 lipids content of the complexes, the distribution of fatty acids in the complexes and the affinity of
330 complexes at oil and water phase. Emulsions stabilized by starch-fatty acid complexes from

331 method I had larger droplet size compared to those stabilized by complexes with same fatty acids
332 from method II. Complexes from method II contained a higher amount of total lipids, larger
333 contact angles than their counterparts from method I, indicating higher level of hydrophobicity.
334 The detachment energy of complexes from method II would be higher, and the resulting
335 emulsions were more stable against coalescences. The distributions of fatty acids in the
336 complexes would also adjust the position of complex particles at the oil-water interface by
337 influencing the partial hydrophobicity of the complexes. The affinity of complexes towards oil
338 and water phase could be affected by the solubility of fatty acids in the oil and water phase. The
339 chain length of fatty acids exhibited small influence on the droplet size of the emulsions
340 stabilized by complexes from method I, while its effects on the emulsion droplets formed by
341 complexes from method II were more profound. The emulsions stabilized by starch-palmitic acid
342 and -stearic acid complexes from method II presented larger droplet size than emulsions
343 stabilized by other starch-fatty acid complexes using the same method. Therefore, the differences
344 in the emulsifying abilities of complexes with similar amount of total lipids might be attributed
345 to the differences in the distributions and chain length of fatty acid in the complexes. For
346 emulsions stabilized by starch-saturated fatty acid complexes, densely packed starch particles
347 could be clearly observed on the oil-water interface of discrete emulsion droplets. These packed
348 particles would form strong steric barriers to further enhance the stability of adjacent droplets
349 against coalescence.[47] In the case of starch-unsaturated fatty acid complexes, no discrete
350 emulsion droplets were observed under microscopic images, indicating no stable emulsions were
351 formed. These results suggested that complexing with unsaturated fatty acids might not be
352 effective to enhance the emulsifying ability of native starch to form Pickering emulsions.

353 **3.4 Influence of heating on emulsion stability**

354 The microscopic images and average droplet size of Pickering emulsions stabilized by different
355 complexes under different heating conditions (60, 80, 100 °C) were depicted in **Figure 3**. Studies
356 have suggested that Pickering emulsion stabilized by starch granules remained stable under heat
357 treatment, which led to partial gelatinized starch granules at the interface to protect the integrity
358 of the emulsions droplets.[24, 48] The droplet size measurements indicated that the heated emulsion
359 droplets were slight higher after heat treatment. And the emulsion droplets size increased with
360 increased temperature during treatment. The microscopic images showed that swelling of intact
361 starch granules were located at the oil-water interface. At heat temperature of 100 °C, the starch-
362 fatty acids complex granules still remained particle status except a noticeable swelling of these
363 particles was observed. The resulting emulsions were still stable with increase in the droplet size
364 resulting from starch swelling after heating. The superior stability of emulsion against heat
365 treatment could be attributed to the relatively high gelatinization temperature of high-amylose
366 starch (70~108 °C).[18] Furthermore, the gelatinization temperature of starch-fatty acid complexes
367 would be further increased due to the formation of amylose-fatty acid crystalline complex, which
368 had high melting temperature of larger than 100 °C.[49] The swelling of starch particles under heat
369 treatment in starch stabilized Pickering emulsions was found to provide better barrier properties
370 and protect the integrity of droplets under freezing and freeze-drying.[24, 48] It suggested that these
371 heated Pickering emulsions might have potential applications as delivery and encapsulation
372 systems of lipophilic compounds in food and pharmaceutical products.

373 **3.5 Encapsulation of PMFs in starch-fatty acid complexes stabilized Pickering emulsions**
374 **and their digestion profile under simulated small intestinal digestion**

375 Since starch-fatty acid complexes prepared using method II seemed to exhibit better
376 emulsifying ability, Pickering emulsion systems stabilized by these complexes were used to

377 encapsulate two major PMFs (5-demethyltangeretin and 5-demethylnobiletin). The oil/water
378 ratio was set at 5:4 to increase the oil faction in the emulsions and decrease the creaming effect.
379 The bulk images of PMFs loaded emulsions stabilized by starch-fatty acid complexes with
380 different fatty acids were presented in **Figure 4(i)**. And the microstructures of these emulsions
381 before and after *in vitro* digestion process were depicted in **Figure 4(ii)**. After encapsulating with
382 PMFs, stable emulsions with no creaming effects were formed. The emulsions exhibited light
383 yellow color coming from PMFs. Discrete emulsion droplets covered with complex granules
384 were observed under optical microscope. The *in vitro* small intestinal digestions of these
385 emulsions were characterized using a pH-stat lipolysis model. The release of fatty acids vs
386 digested time, lipase activity during hydrolysis and bioaccessibility of PMFs after lipolysis were
387 monitored and presented in **Figure 5**. Also, the structure of digestion buffer at the end of
388 lipolysis was observed under optical microscope, seen in **Figure 4(ii)**. After the lipolysis process,
389 the starch-fatty acid complexes remained their intact granular structure despite the fatty acid
390 chain length used, which indicated that starch-fatty acid complexes were resistant to the
391 hydrolysis of amylase (coming from the pancreatin suspension). Compared to emulsions
392 stabilized by complexes with fatty acids (C14:0, C16:0, C18:0), the extent of lipolysis of starch-
393 lauric fatty complexes stabilized emulsions was slower and lower. And its lipase activity
394 recorded was the lowest. The lipolysis progress is the hydrolysis of oil droplets under actions of
395 lipase and gradual detachment of hydrolyzed products (mainly monoglycerides and fatty acids)
396 from the oil droplet surface for further hydrolysis.[50] The better barrier properties of starch-lauric
397 acid complex against lipase hydrolysis might be resulted from the higher lipid content of the
398 complexes. These complexes covering the surface of oil droplets would restrict the interactions
399 between lipase and oil droplets since the fatty acids in the complexes were hard to be removed

400 from the surface. The extent of lipolysis resulting from different starch-fatty acid complexes used
401 affected the release of PMFs during the digestion process. The bioaccessibility of PMFs during
402 lipolysis was the lowest and the highest in emulsions stabilized by starch-lauric acid complex
403 and starch-palmtic acid complex, respectively. It is worth noting that the bioaccessibility of
404 PMFs in these formulations (bioaccessibility, 22%~52%) is much higher than that in previous
405 lecithin-based emulsions (bioaccessibility of 5-demethyltangeretin, < 1%), [38] which highlights
406 the possible high efficiency of starch-fatty acid complex stabilized Pickering emulsion as
407 delivery vehicle for lipophilic bioactive compounds.

408 **3.6 Effect of starch swelling from heating on lipolysis of PMFs loaded-Pickering emulsions**
409 **during simulated small intestinal digestion**

410 The *in vitro* digestion of Pickering emulsions stabilized by different starch-fatty acid
411 complexes suggested that the barrier properties of emulsions using starch-lauric acid complexes
412 were better than using other complexes. Hence, starch-lauric acid complexes stabilized
413 emulsions were treated with heating process to further investigate the effect of starch swelling on
414 the barrier properties. The bulk images of PMFs loaded emulsions stabilized by starch-lauric acid
415 complexes after heating at different temperatures (60, 80, 100 °C), the microstructure of these
416 emulsions before and after lipolysis process were presented in **Figure 6**. After heating treatment,
417 a slight creaming effect was observed in the emulsions. However, these emulsions still remained
418 stable. Discrete emulsion droplets were observed under optical microscope. With the increasing
419 of heating temperature from 60 to 100 °C, a noticeable swelling of starch granules was observed,
420 especially at the temperature of 100 °C. The swelling of starch granules would make these
421 granules more susceptible to hydrolysis of amylase. As depicted in **Figure 6**, the complexes
422 remained granular structure after lipolysis process, indicating that these complexes possessed

423 resistance to amylase digestion even after certain heat treatment. For emulsions without heat
424 treatment, there would be space between individual granules at the oil-water interface even at
425 close packing. As the result, the oil droplets would be easily accessible for lipase. The partially
426 gelatinized complex granules would form a thickness layer around the emulsion droplets, which
427 made them more impermeable for lipase activity as compared to non-heated emulsions. The
428 digestion profile of these emulsions, lipase activity and bioaccessibility of PMFs after lipolysis
429 process were presented in **Figure 7**. Compared to non-heat emulsions, the extent of lipolysis was
430 slower and lower when the emulsions were heated at 60 and 80 °C. The lipolysis rate of
431 emulsions was similar to that of non-heat emulsions when the heating temperature increased to
432 100 °C. Accordingly, the lipase activity dropped to the lowest at 60 °C. It then slightly increased
433 after heat process of 80 °C, which was still much lower than non-heat samples. These
434 phenomena could be interpreted as higher barrier properties of swelling starch to cover oil
435 droplet surface resulting from heating process.[24] Similar reduction in the lipase activity resulted
436 from heating process was observed in octenyl succinic anhydride (OSA) modified quinoa starch
437 stabilized Pickering emulsions.[24] The release of PMFs during lipolysis was reduced at heating
438 temperature of 60 and 80 °C due to improved barrier properties (**Figure 7**). However, the
439 enhanced barrier effect from swelling starch was not observed in emulsions with heat treatment
440 of 100 °C, which might be attributed to the high level of starch gelatinization resulting in the
441 breakdown of some starch granular structure, and the more susceptible of gelatinized starch
442 toward hydrolysis of amylase in the digestion media.

443 **4 Conclusions**

444 V-amylose complexes were formed by complexing starch and different fatty acid chain
445 lengths using two hydrothermal treatment methods. *In vitro* digestibility study suggested that

446 there complexes exhibited enzymatic resistance compared to native starch. The hydrophobicity
447 properties of starch were improved when complexing with fatty acids. With improved affinity
448 towards oil and water phases, starch complexed with saturated fatty acids of chain length ranging
449 from 12 to 18 were able to form stable Pickering emulsion and exhibited different emulsifying
450 efficiencies. However, the emulsifying ability of complexes with unsaturated fatty acid (C18:1)
451 was poor. The addition of fatty acids into the starch retarded the gelatinization of starch.
452 Emulsions stabilized by these complexes were stable under heat treatment of 60, 80 and 100 °C.
453 Starch-fatty acid complexes stabilized Pickering emulsions were used to encapsulate PMFs (5-
454 demethyltangeretin and 5-demethylnobiletin). The *in vitro* lipolysis of PMFs loaded emulsions
455 suggested that starch-lauric acid complex provided better barrier properties during lipolysis
456 process compared to other complexes. Heating treatment of emulsions under 60 and 80 °C
457 improved the barrier properties of the emulsions due to the swelling of starch. The lipase activity
458 during lipolysis reached a minimum at heat treatment of 60 °C. But this enhanced barrier effect
459 was disappeared when the heating temperature was 100 °C since the partial gelatinization of
460 starch was more severe. These results show the potential to create resistant starch based emulsion
461 encapsulation systems with controlled barrier properties using heating process. The starch-fatty
462 acid complex stabilized Pickering emulsions could be used as encapsulation and delivery system
463 with high heat stability and controlled release for lipophilic bioactive compounds for application
464 in various food and pharmaceutical products.

465

466 **Conflicts of interest**

467 We have no conflict of interest in this research.

468

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543
544
545

546 **Figure Captions**

547

548 **Figure 1.** Vessels containing emulsions stabilized by starch-fatty acid complexes (O/W ratio 1:1).
549 A. 12 wt% starch-fatty acid complexes prepared using method I (From left to right: C12:0, C14:0,
550 C16:0, C18:0, C18:1). B. 12 wt% starch-fatty acid complexes prepared using method II (From
551 left to right: C12:0, C14:0, C16:0, C18:0, C18:1).

552 **Figure 2.** Microscopic images and droplet sizes of emulsions stabilized by different starch-fatty
553 acid complexes (12 wt% starch complex, o/w 1:1). The solid bars in microscopic images
554 correspond to the length of 100 μm.

555 **Figure 3.** Microscopic images and droplet sizes of emulsions stabilized by different starch-fatty
556 acid complexes after different heat treatments (12 wt% starch complex, o/w 1:1). The solid bars
557 in microscopic images correspond to the length of 100 μm.

558 **Figure 4.** (i) Bulk images and (ii) Microscopic images of PMF-loaded different starch-fatty acid
559 complexes stabilized emulsions before (1) and after lipolysis (2) (12 wt% starch-fatty acid
560 complexes, o/w 5:4). From A to D: C12:0, C14:0, C16:0, C18:0.

561 **Figure 5.** Extent of lipolysis, lipase activity and bioaccessibility of PMFs in different starch-fatty
562 acid complexes stabilized emulsions.

563 **Figure 6.** Microscopic images of PMF-loaded starch-fatty acid complexes stabilized emulsions
564 with different heat-treatments before (1) and after lipolysis (2) (12 wt% starch-lauric acid
565 complexes, o/w 5:4). From A to C: 60, 80,100 °C.

566 **Figure 7.** Extent of lipolysis, lipase activity and bioaccessibility of PMFs in starch-fatty acid
567 complexes stabilized emulsions after different heat treatments.

568

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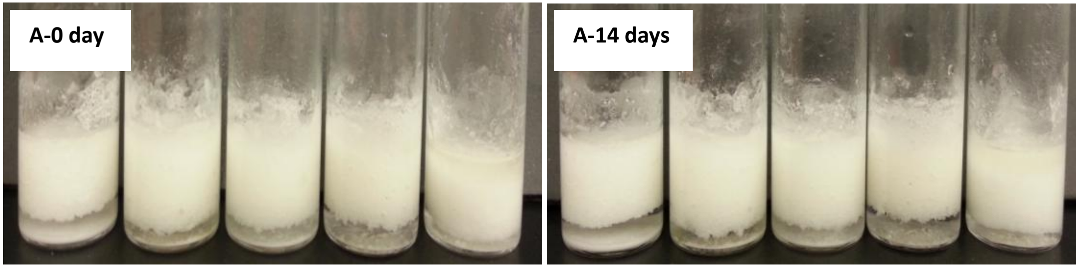

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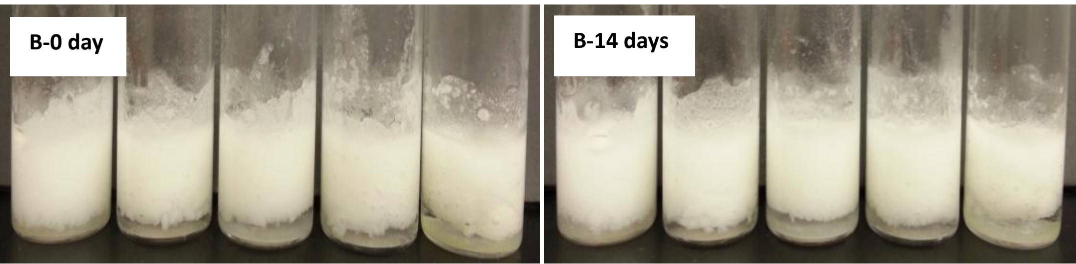

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572 **Figure 1.**

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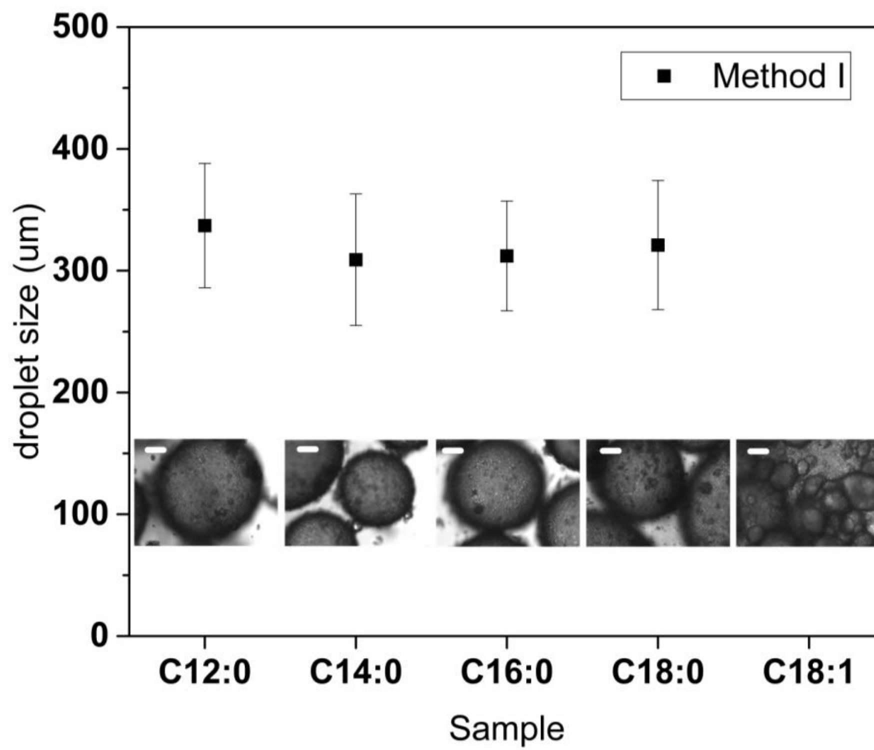

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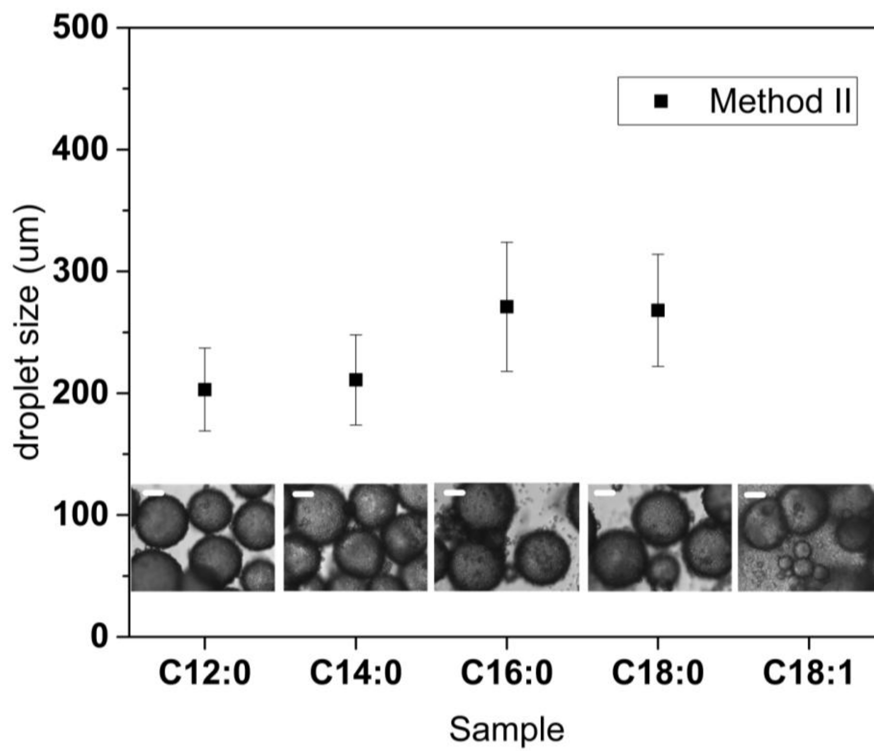

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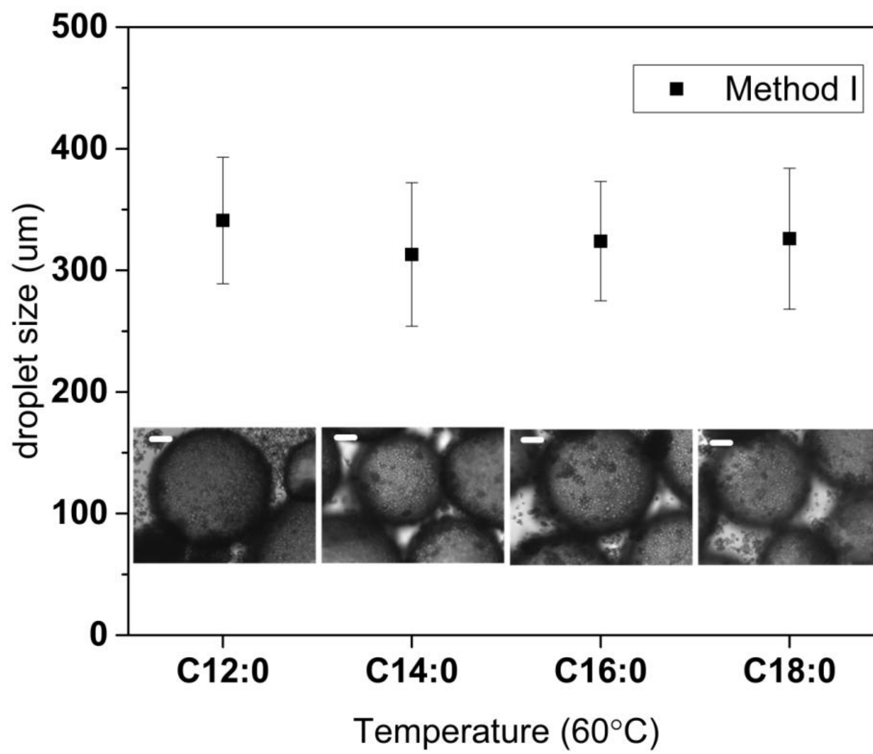
576 **Figure 2.**



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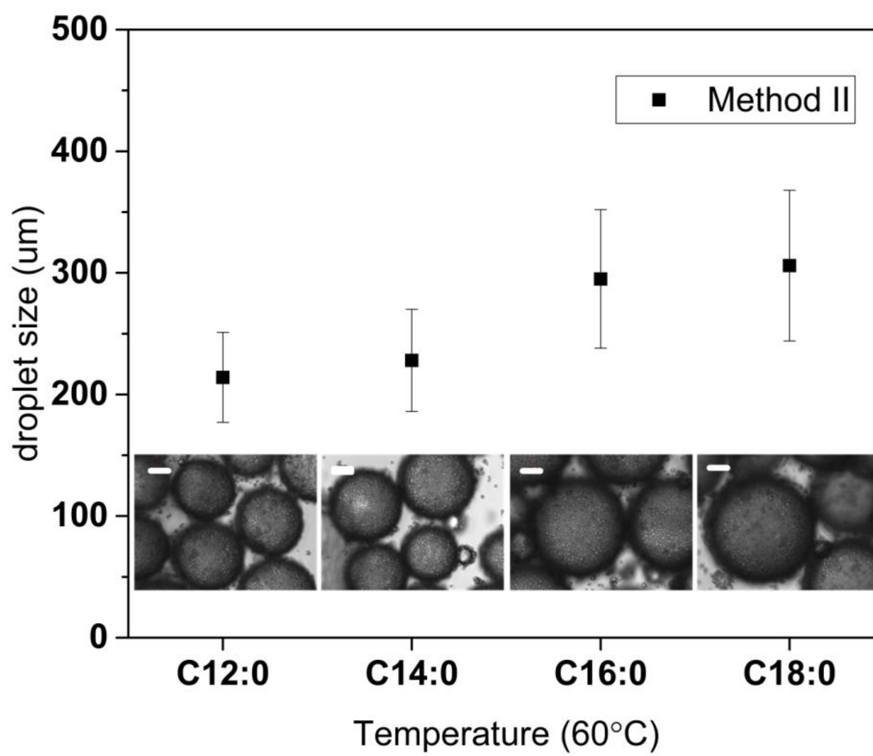

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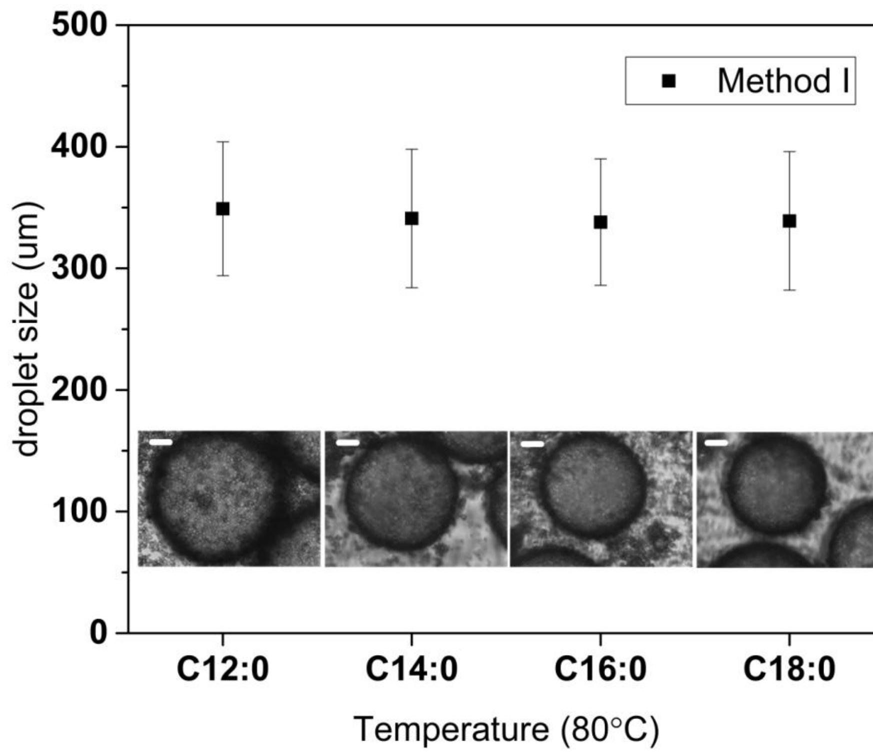

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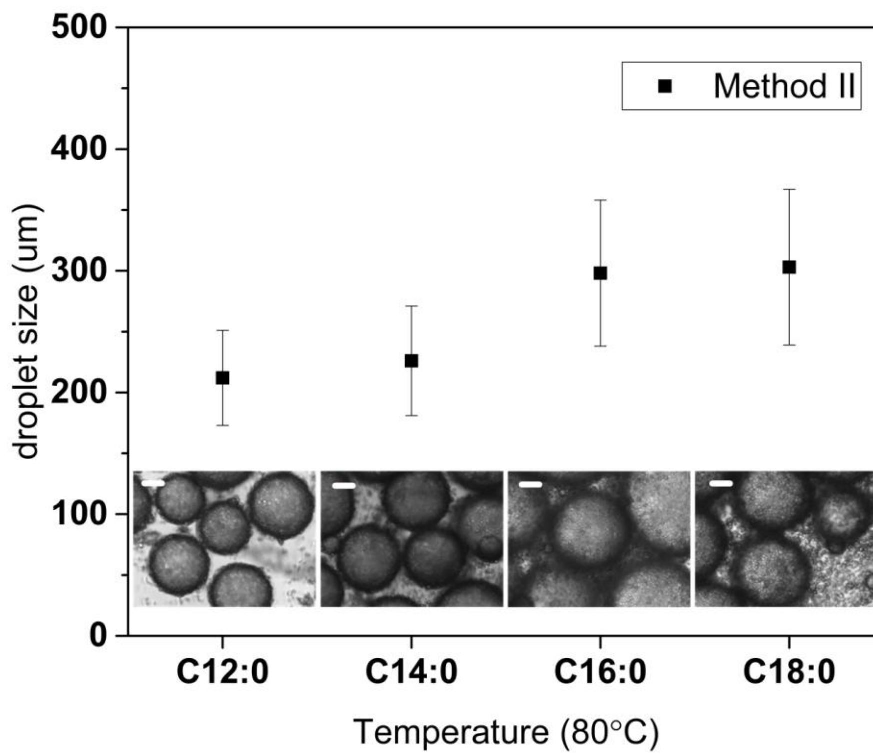

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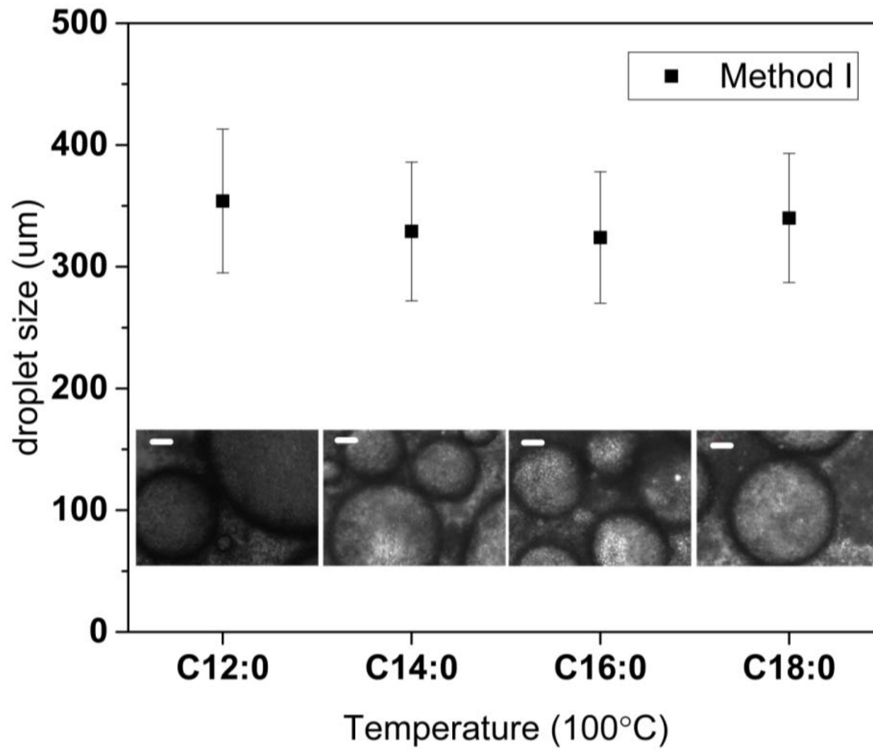

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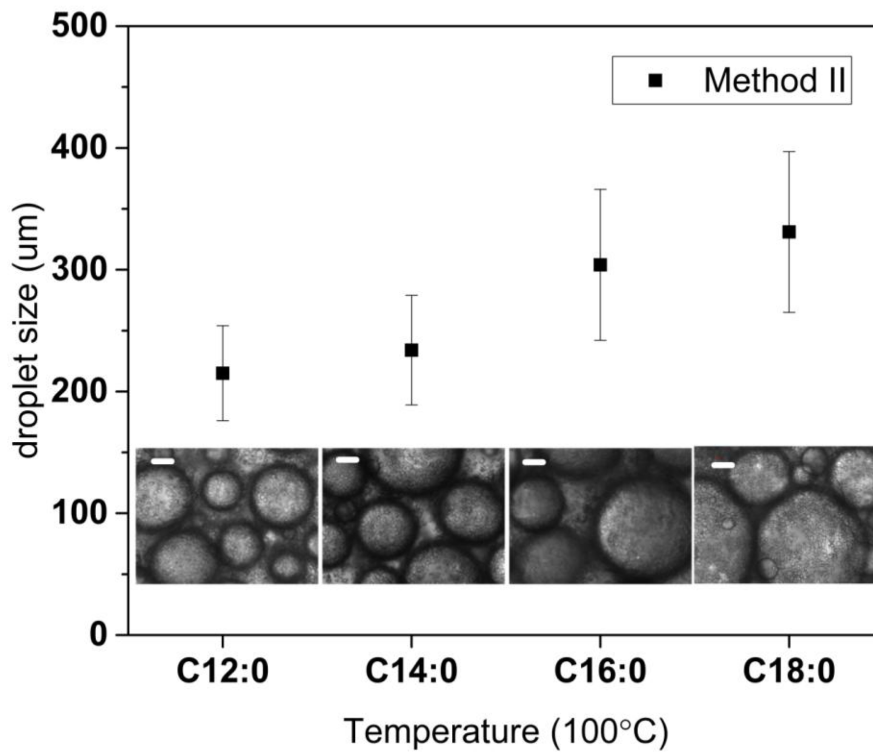

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583 **Figure 3.**

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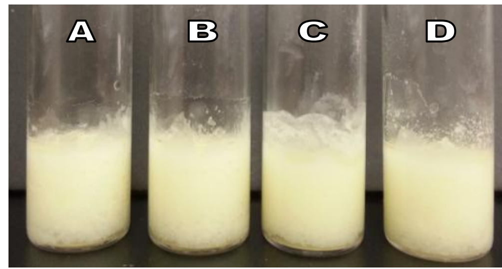
(i)



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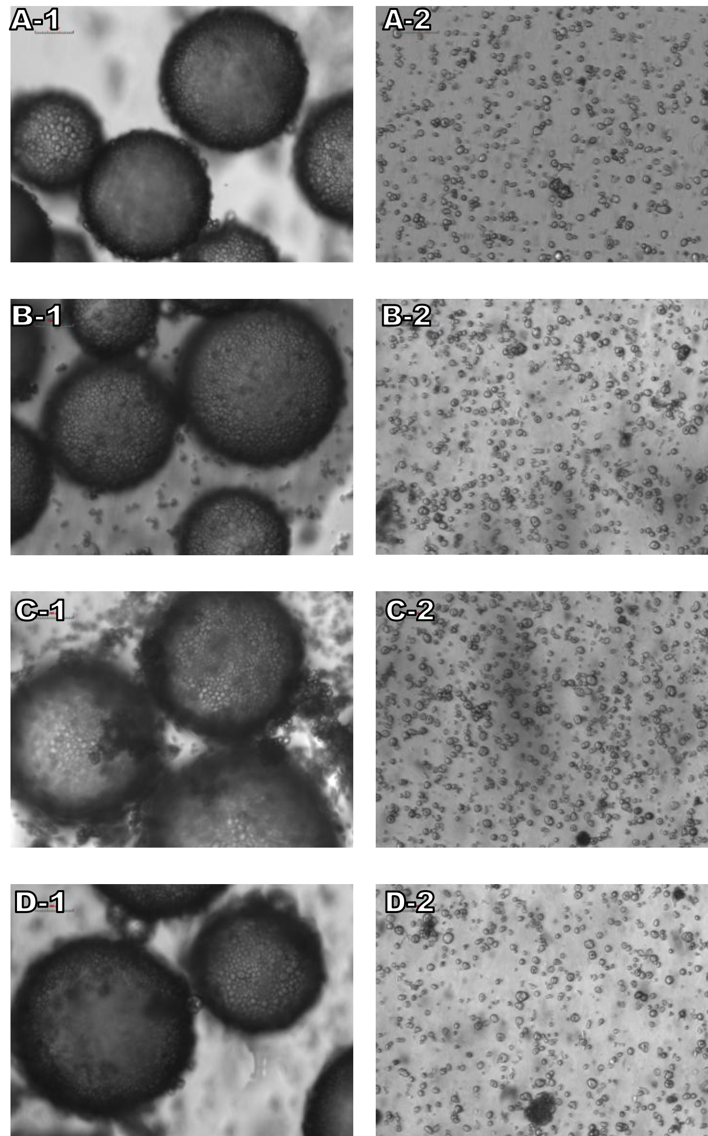
(ii)



586 100 um

587 **Figure 4.**

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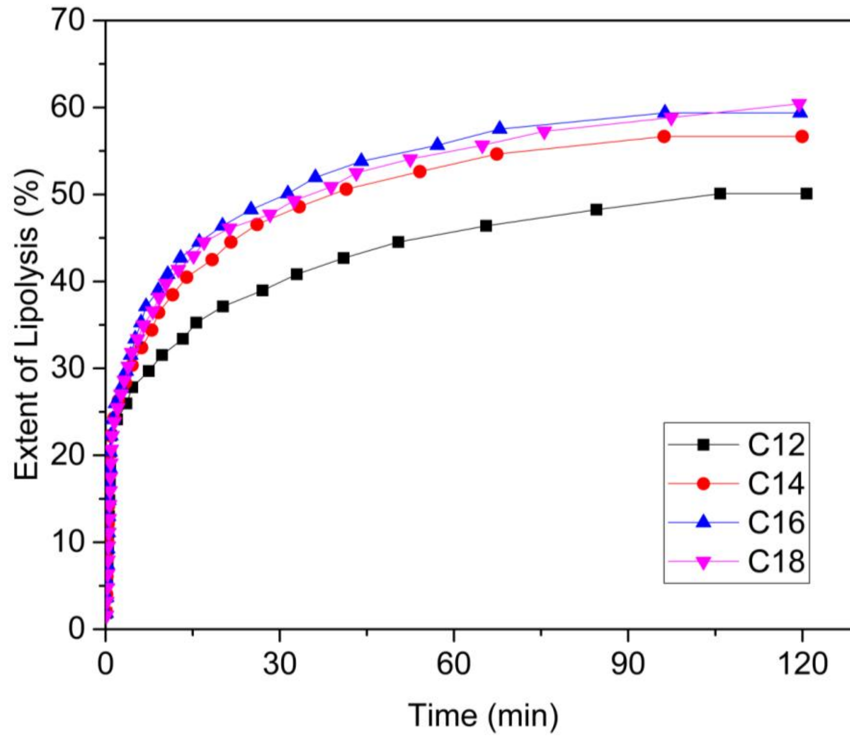

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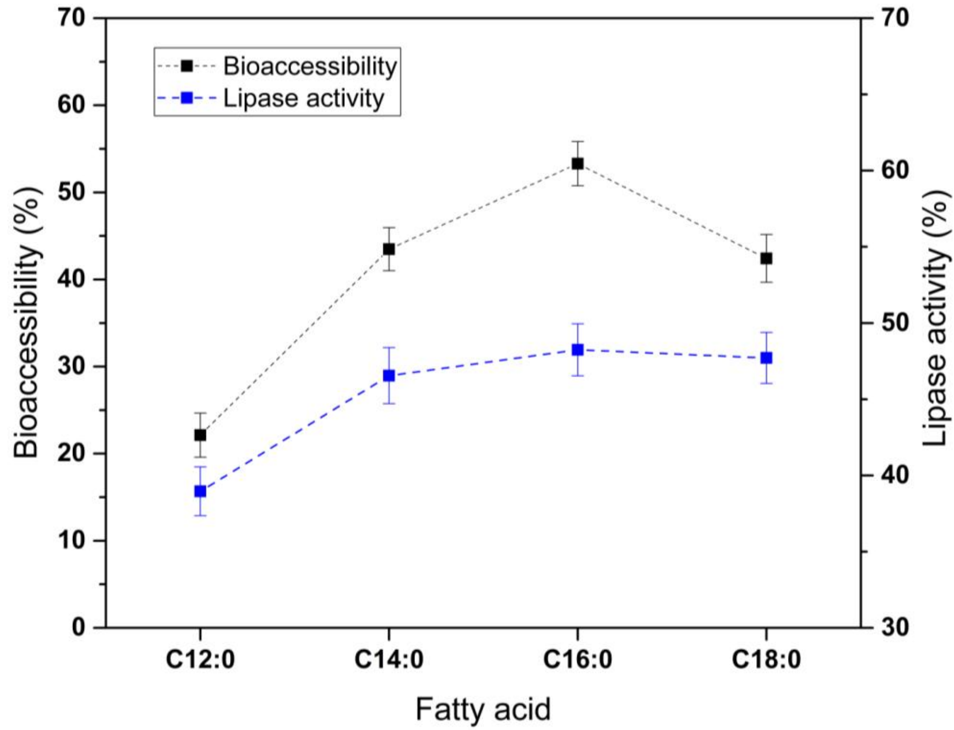

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591 **Figure 5.**

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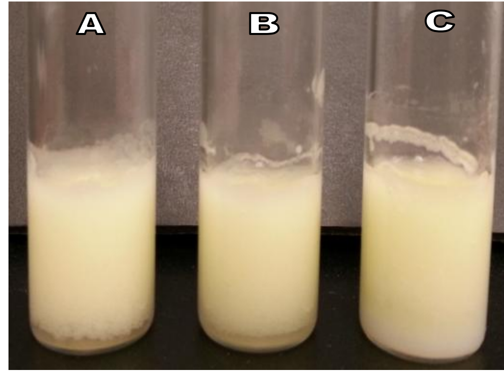

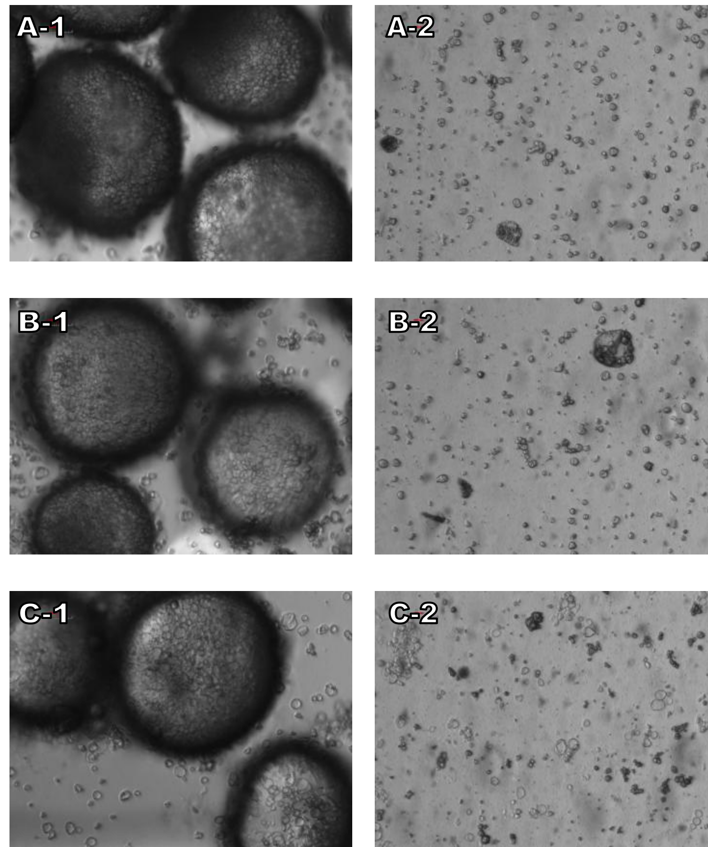

594 100 um

595 **Figure 6.**

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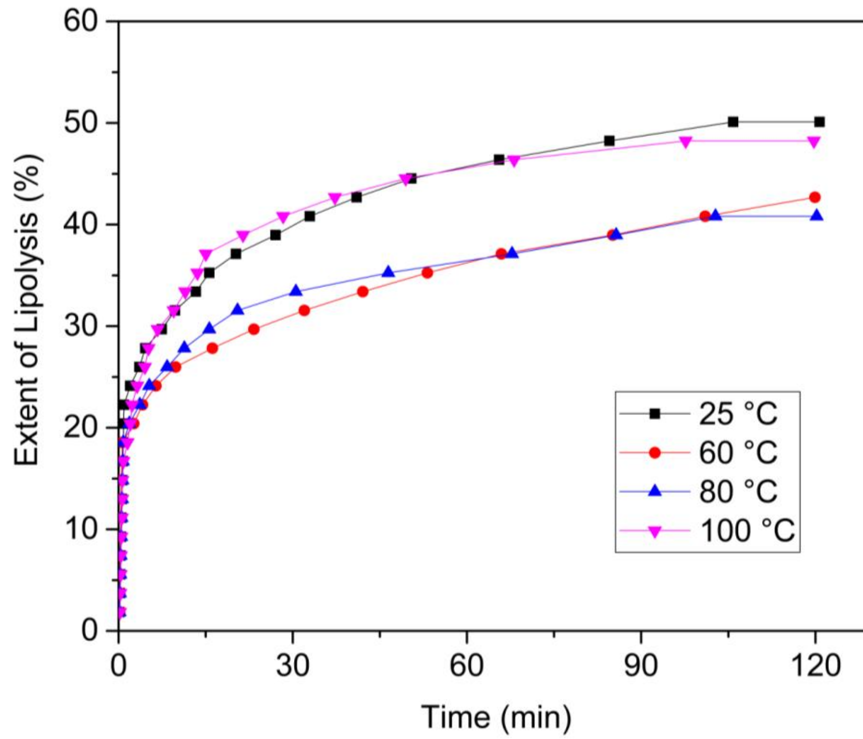

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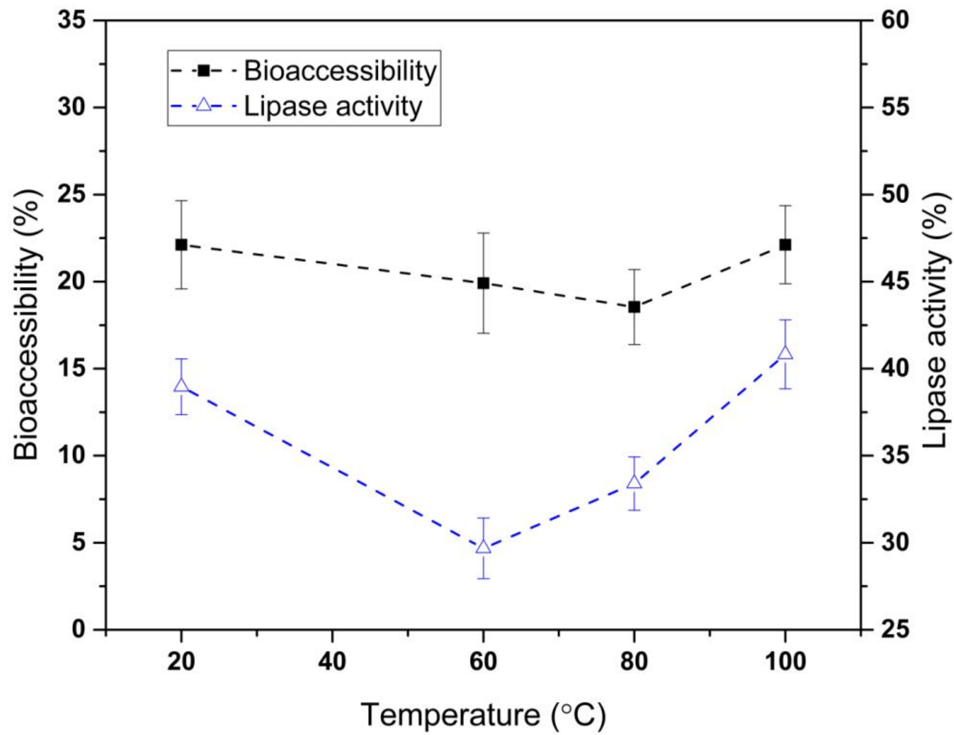

598

599 **Figure 7.**

600

601 **Table 1.** Lipid content of native starch and starch-fatty acid complexes.

Sample[a]	Lipid content (g/100 g of dry starch sample)		
	acid hydrolyzed (total lipids, T)	petroleum ether extracted (free lipids, F)	T – F (complexed lipids)
Native starch	0.92 ± 0.03a	0.52 ± 0.07a	0.40
Method I-C12:0	2.84 ± 0.18bc	1.13 ± 0.47bc	1.71
Method I-C14:0	3.35 ± 0.32c	1.68 ± 0.13d	1.67
Method I-C16:0	3.47 ± 0.29d	1.88 ± 0.36e	1.59
Method I-C18:0	3.35 ± 0.47c	1.84 ± 0.22de	1.51
Method I-C18:1	2.41 ± 0.25b	0.92 ± 0.19b	1.49
Method II-C12:0	3.74 ± 0.31e	1.33 ± 0.38bc	2.41
Method II-C14:0	3.58 ± 0.42d	1.57 ± 0.16cd	2.01
Method II-C16:0	3.56 ± 0.15d	1.71 ± 0.24d	1.85
Method II-C18:0	3.50 ± 0.19d	1.72 ± 0.28d	1.78
Method II-C18:1	2.56 ± 0.23b	0.99 ± 0.11b	1.57

602 [a]Values in the same column with different superscript are significantly different ($p<0.05$).

603

604 **Table 2.** Contact angle of starch-fatty acid complexes.

Sample[a]	Contact angle (°)
Native starch	46.1 ± 1.6a
Method I-C12:0	78.6 ± 2.1c
Method I-C14:0	79.4 ± 1.6cd
Method I-C16:0	81.2 ± 1.7cd
Method I-C18:0	78.5 ± 2.2c
Method I-C18:1	62.3 ± 2.4b
Method II-C12:0	82.6 ± 1.7d
Method II-C14:0	82.1 ± 2.1cd
Method II-C16:0	81.7 ± 1.9cd
Method II-C18:0	80.9 ± 2.4cd
Method II-C18:1	64.2 ± 1.8b

605 [a]Values in the same column with different superscript are significantly different ($p<0.05$).

606

607

608 **Table 3.** RDS, SDS, and RS contents in the cooked starch-fatty acid complexes[a].

Sample[b]	RDS (%)	SDS (%)	RS (%)
Native starch	75.2 ± 1.4e	6.5 ± 1.5d	18.8 ± 0.8d
Method I-C12:0	61.0 ± 0.6d	13.4 ± 0.9a	25.7 ± 0.5a
Method I-C14:0	60.2 ± 1.4cd	13.5 ± 0.8a	26.4 ± 1.1ab
Method I-C16:0	59.3 ± 1.2c	13.8 ± 0.7a	26.9 ± 1.3b
Method I-C18:0	59.5 ± 1.5c	13.3 ± 1.1a	27.2 ± 1.6b
Method I-C18:1	58.2 ± 1.3bc	14.7 ± 0.6bc	27.1 ± 0.9b
Method II-C12:0	58.8 ± 1.7bc	14.0 ± 0.7ab	27.2 ± 0.9b
Method II-C14:0	57.4 ± 1.3b	13.7 ± 0.8a	28.9 ± 1.6c
Method II-C16:0	56.8 ± 1.6b	14.2 ± 1.1ab	29.0 ± 1.5c
Method II-C18:0	56.3 ± 1.2ab	14.4 ± 0.9ab	29.3 ± 1.7c
Method II-C18:1	55.8 ± 1.4a	15.6 ± 0.8c	28.6 ± 0.8bc

609 [a]RDS, SDS, RS refer to rapidly digestible starch, slowly digestible starch, and resistant starch.
610 [b]Values in the same column with different superscript are significantly different ($p<0.05$).
611

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Graphic for table of contents

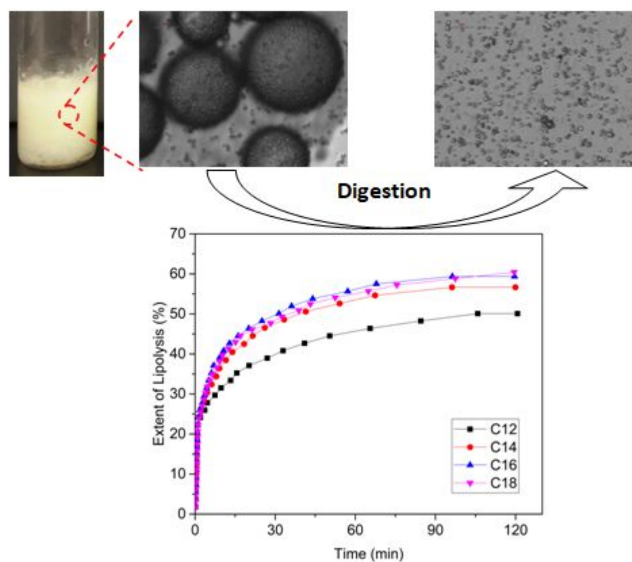

Figure: Digestion of Pickering emulsions stabilized by starch-fatty acid complex.