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1 “Declaration of interest: none”

2 **Spinach leaf and chloroplast lipid: A natural rheology modifier**
3 **for chocolate?**

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13

14 **Abstract**

15 In this study the possibility of replacing current surfactants in chocolate formulations with
16 natural lipids extracted from spinach leaf (SPLIP) or spinach chloroplast (CH.SPLIP) was
17 evaluated. SPLIP and CH.SPLIP were extracted with chloroform/methanol following enzyme
18 deactivation with hot isopropanol. Results showed a higher extraction yield for SPLIP while
19 glycolipids were more concentrated in CH.SPLIP. Sugar/oil suspensions with dispersed
20 volume fractions of 0.28, 0.33 and 0.37 containing 0.1 % to 0.7 % (w/w) surfactant (SPLIP,
21 CH.SPLIP, lecithin and PGPR as commercial references) based on oil phase were prepared
22 and analyzed in shear rheology. Apparent viscosity at 40 s⁻¹ was significantly lower for the
23 natural surfactants compared to lecithin at 0.5 to 0.7 % (w/w) addition. With regard to yield
24 stress, taken as the shear stress at 5 s⁻¹, both natural surfactants showed comparable
25 performance to PGPR at 0.3 % to 0.7 % addition. As SPLIP and CH.SPLIP behaved similar

26 (p > 0.05), SPLIP, due to higher extraction yield, would be the preferred choice for
27 application in chocolate matrices.

28

29 *Keywords:* Chocolate, suspension rheology, lecithin, polyglycerol polyricinoleate (PGPR),
30 interfacial tension, natural surfactant

31

32 **1. Introduction**

33 Chocolate represents a high internal phase volume suspension with sugar and cocoa
34 particles suspended in cocoa butter (CB). The rheological properties of chocolate are not only
35 important in manufacturing steps (Servais, Ranc, & Roberts, 2003) but also to give good
36 quality eating properties (Beckett, 2008). Chocolate is characterized by a shear thinning
37 viscosity behavior with yield stress. The yield stress denotes the transition between pseudo-
38 solid and pseudo-liquid behavior, and it can also be understood as the minimum shear stress
39 at the first evidence (onset) of flow (Doraiswamy et al., 1991). The shear thinning properties
40 are important for pumping and sensory characteristics (Beckett, 2008; Goncalves & Lannes,
41 2010). The International Office of Cocoa (IOC) recommends the characterization of the
42 rheological properties of chocolate between 2 and 50 s⁻¹ by ramping shear rate up and down
43 in 3 min respectively, with one minute holding at 50 s⁻¹. The whole procedure should be
44 preceded by a pre-shear step at 5 s⁻¹ for 5 min (Afoakwa, Paterson, Fowler, & Vieira, 2009;
45 Servais et al., 2003).

46 The rheological properties of chocolate are influenced by the interactions between the
47 dispersed solid sugar and cocoa particles in the CB continuous phase. Sugar particles have a
48 hydrophilic surface and, therefore, are prone to aggregate if no surfactants added to the
49 lipophilic continuous phase. Aggregation leads to entrapment of CB thereby apparently
50 increasing the particle volume fraction, increasing yield stress and apparent viscosity. Hence,

51 a surfactant is added to coat the surface of the sugar particles so they disperse well in the
52 continuous CB phase.

53 Depending on the type of chocolate, commercial chocolate contains around 29 – 40 %
54 (w/w) fat (Beckett, 2009) and 0.3 – 0.5 % (w/w) surfactant (Beckett, 2008). The most
55 commonly used surfactants are lecithin and polyglycerol polyricinoleate (PGPR). Lecithin
56 promotes apparent viscosity reduction while PGPR decreases the yield stress without
57 significantly affecting the apparent viscosity. Therefore, these two surfactants are often
58 combined to obtain the desired product rheology (Schantz & Rohm, 2005). Contrary to
59 lecithin, naturally produced from the by-product of oil refining (van Nieuwenhuyzen, 2010),
60 PGPR is chemically synthesized through polyesterification of glycerol and ricinoleic acid
61 from castor oil (Christiansen, 2014). Both surfactants are assigned an E-number and are thus
62 not considered clean-label. Consumers are often familiar with lecithin and likely to accept its
63 presence in processed foods. PGPR on the other hand creates negative associations due to its
64 complicated name and the fact that it is a synthetic material appears to be widely known
65 among health-conscious consumers (Osborn, 2015).

66 Efforts to replace PGPR with a natural alternative date back some 30 years when a
67 patent on the polar lipid fraction of oats, specifically the glycolipid fraction, as a low shear
68 viscosity reducing agent in chocolate was published (Evans, Jee, Sander, Smith, & Gibson,
69 1991). Depending on oat species and variety, 10 – 34 % (w/w) of the total oil was reported to
70 constitute polar lipids, mainly glycolipids (5 – 15 % (w/w)) and phospholipids (5 – 26 %
71 (w/w)) (Doehlert, Moreau, Welti, Roth, & McMullen, 2010; Sahasrabudhe, 1979; Youngs,
72 Puskulcu, & Smith, 1977). The main components of the glycolipid fraction were identified as
73 galactolipids including digalactosyl diacylglycerol (DGDG), 41.5 % (w/w), and
74 monogalactosyl diacylglycerol (MGDG), 18.5 % (w/w). Other glycolipids were present at a
75 level of less than 10 % (w/w) (Sahasrabudhe, 1979). The phospholipid fraction was identified

76 as containing phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and a minor
77 fraction of phosphatidyl inositol (PI) (Doehlert et al., 2010; Kaimainen et al., 2012). To the
78 best of the authors' knowledge, this research on oat oil has not been validated with alternative
79 natural lipid extracts rich in the main oat lipid components, which motivated the present
80 study.

81 MGDG and DGDG are also abundantly available in photosynthetic plants, in
82 particular spinach (*Spinach oleracea L.*) which is therefore often selected as an exemplary
83 photosynthetic plant in studies related to plant based polar lipids (Allen, Good, Davis,
84 Chisum, & Fowler, 1966; Douce, 1974; Douce, Holtz, & Benson, 1973; Jaime et al., 2015).
85 Other lipids found in spinach include the sulfolipid sulfoquinovosyl diacylglycerol (SQDG)
86 (Christie, 2012), and the phospholipids phosphatidylglycerol (PG) and phosphatidylcholine
87 (PC) (Mazliak, 1977). Galactolipids are neutral lipids while SQDG and PG each carry one
88 negative charge in their head group (Dörmann & Benning, 2002).

89 This study presents data on the composition and efficacy as fat based suspension
90 rheology modifier of lipid extracted from both spinach leaf and spinach chloroplast. The
91 polar lipid fraction of either has been reported to show little compositional difference
92 (Dörmann, 2013; Wintermans, 1960), but extraction yield will be higher from isolated
93 chloroplast due to their enrichment in lipids. Chloroplast isolation comprises an additional
94 processing step, hence, in potential future commercial application, leaf lipid may be of higher
95 value despite the lower extraction yield. Here, the performance of both lipids was compared
96 to lecithin and PGPR as commercially applied surfactants, using sugar/oil suspensions, a
97 common fat based food suspension model of chocolate.

98

99 **2. Materials and methods**

100 2.1. Materials

101 Fresh spinach leaves, icing sugar and sunflower oil were bought from a local
102 supermarket. The moisture content of the fresh spinach leaves was, on average, 94.0 ± 0.2
103 g/100 g (wet basis), determined by oven drying to constant weight at 105 °C. Icing sugar was
104 used to prepare the sugar/oil suspensions and its properties relevant to this study are reported
105 in section 2.8. Sunflower oil, as the suspension medium, was purified to remove any surface
106 active molecules by adsorption to magnesium silicate (Florisil®, Sigma-Aldrich, Dorset,
107 UK), as described in section 2.6. PGPR 90 was provided by Danisco (Kettering, UK), lecithin
108 was from ADM (Hull, UK) and CB was from Barry Callebaut (Banbury, UK). Extraction
109 solvents were chloroform (Sigma-Aldrich, UK), methanol (Fisher Scientific, Loughborough,
110 UK) and isopropanol (Fisher Scientific, Loughborough, UK). Other materials included
111 sodium chloride (Sigma-Aldrich, USA), sucrose (Sigma-Aldrich, USA) and deionized water.
112 Further materials used by an external laboratory for lipid analysis are mentioned with the
113 method.

114

115 2.2. Lipid extraction from spinach leaf

116 A heat pre-treatment with hot isopropanol was carried out prior to leaf lipid
117 extraction, to prevent the activity of hydrolytic enzymes, which are easily activated when
118 plant cells are ruptured (Benson, 1964; Fishwick & Wright, 1977; Kates & Eberhardt, 1957).
119 The pre-treatment followed the method of Yao, Gerde, and Wang (2012) where 100 g of
120 fresh leaves was finely homogenized in 300 ml of pre-heated isopropanol (80 °C), using a
121 glass household blender (kMix BLX50BK, Kenwood, UK) for 1 min. The mixture was then
122 poured into a beaker and heated at 80 °C for 20 min while stirring at 400 rpm on a magnetic
123 hot plate stirrer. Treated leaf and solvent containing isopropanol soluble lipids were separated
124 by filtering through three layers of cheesecloth on a Buchner funnel aided by vacuum suction.

125 The filtrate was retained for lipid recovery and combined with the filtrate from the following
126 lipid extraction step.

127 Lipid extraction was following the established method of Folch, Lees, and Sloane-
128 Stanley (1957). The treated leaf collected from the heat pre-treatment procedure was mixed
129 with 240 ml of chloroform/methanol (2:1, v/v) and stirred on a magnetic plate stirrer at 400
130 rpm for 20 min (Folch et al., 1957). Following the filtration procedure as previously
131 described, the extract was filtered and the filtrate was combined with that extracted from the
132 heat pre-treatment. Solvents in the combined extracts were evaporated at 40 °C until almost
133 dry. The extract was then reconstituted with 24 ml of chloroform/methanol (2:1, v/v),
134 transferred into a separatory funnel and 6 ml of NaCl (0.9 %; w/v) solution was added for the
135 final mixture to be close to 8:4:3 (v/v) of chloroform/methanol/NaCl (Folch et al., 1957). The
136 extract was left to stand for at least 1 h until complete separation of the two liquids was
137 visible. The upper phase contained all of the non-lipid substances and negligible amounts of
138 lipids while the lower phase contained essentially all of the tissue lipids (Folch et al., 1957).
139 The lipid phase was then transferred into a clean, pre-weighed flask. The solvent was
140 evaporated at 40 °C and the extracted lipids were weighed gravimetrically. The collected
141 lipids were re-dissolved in an amount of chloroform (10 times the weight of extracted lipids)
142 and then stored at -80 °C until further use.

143

144 2.3. Lipid extraction from spinach chloroplast

145 Chloroplasts were isolated from spinach leaf following a procedure introduced by
146 Gedi et al. (2017) with slight modification. Seventy grams of fresh leaf was homogenized in a
147 household blender with 210 ml of 0.3 M aqueous sucrose solution for 1 min at room
148 temperature. The slurry was filtered through three layers of cheesecloth and the chloroplasts
149 in the filtrate were isolated by centrifugation (1500 g, 20 min) at 4 °C. The chloroplast pellet

150 was then ready for lipid extraction, following the same protocol as for the leaf, including the
151 pre-treatment with hot isopropanol (see section 2.2). The yield of the chloroplasts was
152 determined gravimetrically by freeze-drying until constant weight. Approximately, the
153 amount of chloroplast obtained was 1 g (freeze-dried) per 100 g of fresh leaf.

154

155 2.4. Lipid analysis by Thin Layer Chromatography (TLC)

156 All experiments for the determination of lipid composition using TLC and gas
157 chromatography (GC) were carried out by an external laboratory (Mylnefield Lipid Analysis
158 at James Hutton Limited, Dundee, UK). Due to the cost involved, samples were analyzed
159 only once.

160 For TLC, the major class of lipids (polar lipids and neutral lipids) were separated
161 using 1-dimensional (1-D) glass HPTLC (high performance TLC) (Silica gel 60 F₂₅₄ plates
162 (Merck, Darmstadt, Germany)), while the polar lipids were separated using 2-dimensional (2-
163 D) glass HPTLC, as follows. A known amount of phosphatidylcholine containing
164 heptadecanoic acid (C17:0PC) as the internal standard was added into the lipid extract to aid
165 analysis with the 1-D TLC plate. The mixture of the lipid sample and the internal standard
166 was spotted (200 µl) onto the 1-D TLC plate and then separated in one direction using
167 70:30:2 (v/v/v) of isohexane/diethyl ether/formic acid solvent mix. The plate was sprayed
168 with primuline and viewed under UV light. The lipid components were then extracted from
169 the silica plate prior to analysis by GC.

170 Polar lipid separation used phosphatidyl ethanolamine containing heptadecanoic
171 acid (C17:0PE) as the internal standard. A small amount of lipid sample was spotted near the
172 corner and separated in two directions. A solvent mixture of 65:25:2.8 (v/v/v)
173 chloroform/methanol/water and 80:12:15:4 (v/v/v/v) chloroform/methanol/acetic acid/water
174 was used as the solvent mixture for the first and second direction, respectively. An identical

175 plate of standards was run at the same time to aid the identification of the spots on the sample
176 plate. The polar lipid fractions, MGDG, DGDG, SQDG and trigalactosyl diacylglycerol
177 (TGDG) were removed from the TLC plate and re-extracted from the silica. A second
178 internal standard (henicosanoic acid, C21:0) was added to all fractions before esterification
179 for GC analysis.

180 The band of TLC adsorbent containing lipids was scraped and put into a glass test
181 tube. About 1 ml of toluene and 2 ml of methanolic sulfuric acid (1 %; v/v) were added into
182 the glass test tube. The mixture was then heated to, and held at, 50 °C for 14 – 16 h. After
183 cooling, it was shaken with 2 ml of isohexane and 5 ml of NaCl solution (5 %; w/v). The
184 solvent was then transferred into a new glass test tube. The previous test tube was shaken
185 with another 2 ml of hexane and the two solvents were combined in the new glass test tube.
186 The combined solvents were shaken with 3 ml of KHCO₃ solution (2%; w/v). The solvent
187 mixture was then transferred into a new tube and 1 ml of toluene was added before blowing
188 off the solvents into dryness with N₂ gas. After that, isohexane and BHT (butylated
189 hydroxytoluene, antioxidant) were added to give a lipid concentration of 5 mg/ml. The fatty
190 acids methyl esters (FAMES) were then ready to be injected into the gas chromatograph for
191 further analysis.

192

193 2.5. Lipid analysis by Gas chromatography (GC)

194 The profile of the fatty acid methyl esters (FAMES) was determined using GC
195 (Agilent 6890, Agilent, USA). The fatty acids were separated using a capillary column (Cp-
196 wax 52CB, 30 mm x 0.25 mm internal diameter x 0.15 µm, Agilent, UK). Hydrogen was
197 used as the carrier gas at the flow rate of 40 ml/ min. The column temperature was initially
198 held at 170 °C for 3 min. The temperature was then increased to 220 °C at 4 °C/min and
199 maintained for 10 min. An amount of 1 µl of sample was injected into a 230 °C inlet with a

200 50:1 split ratio. A flame ionization detector at a temperature of 300 °C was used. The data
201 were processed by integrating the area under the curve and the results are reported as
202 normalized area (%) and mg fraction/g oil.

203

204 2.6. Preparation of oil phases for oil-based suspension system

205 Surfactants (spinach lipids (either leaf or chloroplast), lecithin or PGPR) in
206 sunflower oil solutions were prepared at concentrations of 0.1 %, 0.3 %, 0.5 % and 0.7 %
207 (w/w). The sunflower oil was first purified with 4 % (w/w) magnesium silicate and stirred for
208 30 min at 600 rpm followed by centrifugation at 1700 g for 25 min to remove the silicate. To
209 prevent re-introduction of surface active material due to rancidification, the purified oil was
210 stored in the dark at 4 °C for a maximum of one week. The absence of surface activity within
211 the one week was validated by measuring interfacial tension against water to ensure that it
212 was constant at 30 ± 1 mN/m.

213 The addition of spinach lipid to the purified oil followed a procedure of mixing in
214 chloroform dissolved extract (roughly 1 g extract depending on extract yield in the 10
215 volumes of chloroform, as stated in section 2.2) with purified oil (50 g) in a round bottom
216 flask and mixed by swirling for at least 1 min. This was followed by allowing the chloroform
217 to evaporate at 40 °C. Complete evaporation of the chloroform was checked by mass balance.
218 By diluting with purified oil, the desired spinach lipid concentrations of 0.1 %, 0.3 %, 0.5 %
219 and 0.7 % (w/w) were obtained.

220 Purified oil containing lecithin and PGPR at the same concentrations were prepared
221 by mixing the required amount of either lecithin or PGPR with purified oil (up to 100 g) in a
222 glass beaker by stirring for 24 h on a magnetic stirrer at 600 rpm and room temperature.

223

224 2.7. Dynamic interfacial tension

225 The interfacial tension at the water/oil interface was measured as a function of time
226 with a Drop Shape Tensiometer (PAT-1, Sinterface, Berlin, Germany) for surfactant
227 concentrations of 0 %, 0.001 % and 0.005 % (w/w) in the oil. The highest concentration was
228 limited to 0.005 % (w/w) due to the deep green color of the spinach lipid extract which
229 interfered with the measurement principle (reliance on translucency of non-drop forming
230 fluid). The (lightly green colored) oil phase was added to a cubic glass cuvette and a drop of
231 water, with a cross sectional projection area of 30 mm², was suspended into the oil sample
232 from the tip of a straight capillary of 2 mm outer diameter. The drop was formed in less than
233 one second and its shape was monitored for 900 s by a video camera coupled to a computer.
234 The measurement temperature was 20 °C. The values reported in the results section represent
235 an average of three independent measurements.

236

237 2.8. Particle size distribution of icing sugar

238 The icing sugar used in this study was pre-dried at 60 °C for 24 h under a pressure
239 of 800 mbar using a vacuum oven (Gallenkamp, Fistreem International, Loughborough, UK).
240 The particle size of the icing sugar was analyzed using laser diffraction equipment (Beckman
241 Coulter LS13320, Meritics, Wycombe, UK), fitted with a dry powder module (Beckman
242 Tornado Dry Powder System, Meritics, UK). The distribution was tri-modal and therefore
243 separated into three populations, using the equipment's software. The size boundaries with
244 the respective volume based fraction of the total distribution, as well as the characteristic
245 particle sizes are reported in Table 1.

246 **Table 1**

247 Characteristic size distribution values for the three particle populations of the icing sugar
248 sample used in the sugar/oil suspension systems. The percentage volume differential shows
249 the total amount (in percent) of particles in the particular group of size particle. Reported are

250 the volume based diameter $d_{4,3}$ describing the common mean diameter over the volume
 251 distribution for a monodispersed sample and the volume based characteristic particle sizes for
 252 which 10 %, 50 % and 90 % of the particles were smaller than the size boundary.

Size boundary	0.38 – 1.83 μm	1.83 – 76.43 μm	76.43 – 194.20 μm
<i>Volume (%)</i>	6.43 ± 0.22	80.47 ± 0.06	13.10 ± 0.30
$d_{4,3}$ (μm)	0.97 ± 0.01	26.05 ± 0.16	107.33 ± 3.12
$d_{10,3}$ (μm)	0.53 ± 0.01	5.59 ± 0.07	81.70 ± 0.40
$d_{50,3}$ (μm)	0.91 ± 0.01	21.83 ± 0.25	104.43 ± 2.87
$d_{90,3}$ (μm)	1.55 ± 0.01	54.20 ± 0.18	137.07 ± 5.34

253

254 2.9. Density of icing sugar

255 The density of the icing sugar needed to be known to adjust the phase volume of the
 256 suspensions. It was determined at room temperature using the volume displacement method
 257 based on sunflower oil with the density of $0.92 \pm 0.02 \text{ g/cm}^3$, previously determined with a
 258 density meter (Anton Paar, Germany), as follows. An equal weight of sugar and oil were
 259 mixed together using an impeller stirrer (1000 rpm, 60 min) until well dispersed. Based on
 260 the weight of a known volume of the dispersion the density of the icing sugar was computed
 261 as $1.55 \pm 0.04 \text{ g/cm}^3$. This was comparable with a published value of 1.58 g/cm^3 (Arnold et
 262 al., 2013).

263

264 2.10. Preparation of sugar/oil suspension

265 The effect of the lipid extracts as a rheology modifier in comparison to the
 266 commercial reference surfactants was tested on sugar/oil suspensions with sugar volume
 267 fractions of 0.28, 0.33 and 0.37. The suspensions were prepared by dispersing the appropriate

268 amount of sugar into pre-prepared oil phase, containing surfactant at the desired
269 concentration, with an impeller stirrer (IKA Werke, Staufen, D) operated at 1000 rpm for 60
270 min. To prevent sedimentation of the sugar, the suspensions were then continuously mixed at
271 gentle mixing condition for 24 h using an end-over-end mixer (Reax 2, Heidolph,
272 Schwabach, D) until rheological measurement was performed.

273 The selection of the sugar volume fractions and the design of the suspension
274 preparation protocol was based on published literature (Arnold et al., 2013) that also guided
275 the selection of the rheology protocol applied here (see section 2.11). These authors
276 formulated their sugar in soybean oil suspension at the sugar mass fraction of 0.45, which
277 equates to the sugar volume fraction of 0.33 in our system. Here, 0.4 (0.28) and 0.5 (0.37) as
278 a slightly lower and higher mass (volume) fraction of sugar respectively was included in the
279 experimental design. Therefore, in terms of fat content, the suspensions assessed in this study
280 ranged from 50 to 60 % (w/w). This is higher than in commercial chocolate formulations, 29
281 to 40 % (w/w) as aforementioned, rendering the system less viscous. With a D_{90} of 137 μm , see
282 Table 1, the sugar particles were significantly larger than the D_{90} of around 30 – 40 μm of
283 commercial formulations (Afoakwa, Paterson & Fowler, 2008) and, together with the larger
284 fat content, the lower energy input provided by the overhead mixing set-up compared to
285 industrial chocolate manufacture sufficed to ensure breaking up of sugar aggregates and
286 homogeneous coating of the sugar particles. It is also worth noting that here, in difference to
287 commercial processing, all of the surfactant was present at the beginning of the mixing
288 process.

289

290 2.11. Suspension rheology

291 The rheological properties of the sugar/oil suspensions were evaluated by acquiring
292 shear viscosity curves on a rotational shear rheometer (MCR 301, Anton Paar, Graz, A) fitted

293 with a concentric cylinder geometry (bob diameter of 27 mm, cup diameter of 29 mm, bob
294 length of 40 mm; CC27, Anton Paar, Graz, A). Published protocol (Arnold et al., 2013) was
295 followed and slightly modified by starting the measurement with a pre-shear at 10 s^{-1} for 50 s
296 to improve the reproducibility of the data. Real measurement started by increasing shear rate
297 from 0.01 s^{-1} to 1000 s^{-1} in a logarithmic ramp within 990 s. After stopping the shear for 120
298 s, the shear rate was decreased from 1000 s^{-1} to 0.01 s^{-1} (logarithmic ramp, 990 s). Fifty-one
299 data points were taken on each logarithmic ramp with the measurement time logarithmically
300 decreasing from 100 s at 0.01 s^{-1} to 0.5 s at 1000 s^{-1} , and then increasing again to 100 s at
301 0.01 s^{-1} for the decreasing shear rate ramp. The measurement temperature was $22 \text{ }^{\circ}\text{C}$ as in the
302 published method (Arnold et al., 2013) and results are presented as relative viscosity (the
303 ratio of the measured viscosity to the viscosity of the continuous phase). The addition of any
304 of the surfactants did not affect the apparent viscosity of the sunflower oil and it remained
305 Newtonian. The average apparent viscosity of the oil phases used in this study was $0.060 \pm$
306 $0.002 \text{ Pa}\cdot\text{s}$. Referring to the viscosity curves obtained, the apparent viscosity and yield stress
307 are reported as the apparent viscosity at 40 s^{-1} and the shear stress at 5 s^{-1} of the increasing
308 shear ramp, respectively.

309

310 2.12. Statistical analysis

311 Spinach lipid extraction was carried out in duplicate (two different batches) and each
312 batch was utilized to prepare one set of oil-based suspension phases (SPLIP and CH.SPLIP)
313 at the required concentration. Lecithin and PGPR based oil phases were also prepared
314 independently in duplicate at the required concentrations. Each suspensions prepared from
315 each oil phase was then analyzed in duplicate. A third batch was prepared if unreliable data
316 were obtained. All data are presented as mean values \pm standard deviations of $n = 4$. Mean
317 comparison was carried out using one-way ANOVA. Significant differences between

318 samples were analysed using Tukey HSD (Honestly Significant Different) multiple
319 comparisons test at 95 % confidence level. The software used was IBM SPSS Statistics 22.

320

321 **3. Results and discussion**

322 3.1. Yield of lipid extracts

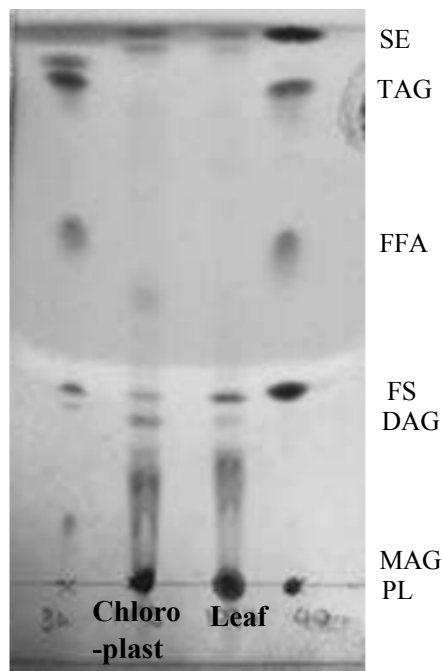
323 The yield of lipid obtained from the leaf (SPLIP) and chloroplast (CH.SPLIP) was
324 14.9 (\pm 4.5) g/100 g dried spinach leaf and 24.0 (\pm 4.6) g/100 g dried chloroplast,
325 respectively. The higher yield from the chloroplast was expected as the lipids in green plant
326 leaf tissue are mostly concentrated in the chloroplast (Nishimura, Graham, & Akazawa,
327 1976). The yield from the leaf was comparable to values reported by Fricker, Duben, Heintze,
328 Panlas, and Zohm (1975) and Yunoki et al. (2009). However, a result reported by Menke
329 (1938) was about 10 % higher than the data reported in this study. As the amount of isolated
330 chloroplasts obtained was about 1 g (dry weight) per 100 g of fresh spinach leaves, 100 g
331 fresh spinach leaves would yield 0.24 (\pm 0.05) g lipids from the isolated chloroplasts. On the
332 other hand, directly extracted lipid obtained from the whole fresh leaves, thereby omitting the
333 chloroplast isolation step, was 0.89 (\pm 0.27) g per 100 g of fresh spinach leaves rendering the
334 direct use of leaf commercially more interesting.

335

336 3.2. Lipid classes

337 The lipid classes in the two spinach extracts were initially identified by separation
338 using the method of 1D TLC, see Fig. 1 for the chromatogram. Lipids detected for both
339 extracts were sterol ester (SE), triacylglycerols (TAG), free fatty acids (FFA), free sterol
340 (FS), diacylglycerols (DAG), monoacylglycerols (MAG) and polar lipids (PL). The polar
341 lipid spot remained at the origin of the plate showing that it was strongly absorbed to the
342 stationary phase. Non-polar lipids eluted and appeared at the end of the chromatogram. Polar

343 lipids were reported to be in abundance in spinach (Dörmann, 2013), explaining their more
 344 intense spots compared to the spots of the other lipid classes.

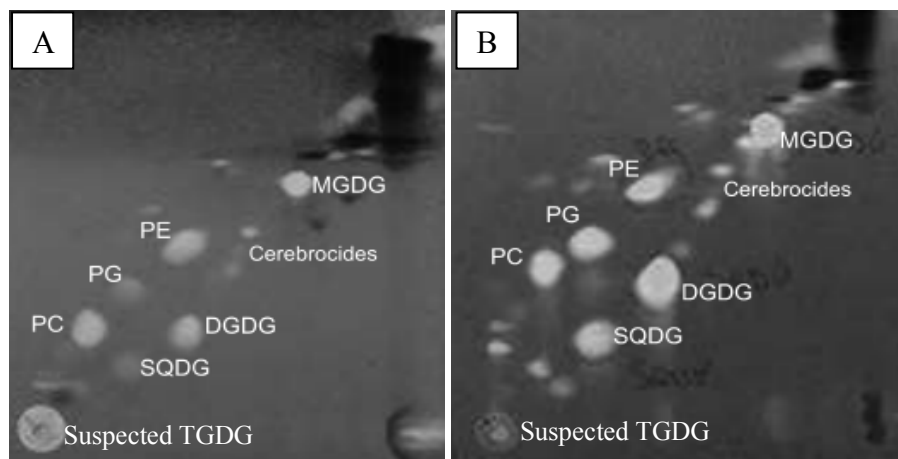


345

346 **Fig. 1.** Separation of lipid from spinach leaf and spinach chloroplast. Abbreviation denote:
 347 SE = sterol ester, TAG = triacylglycerols, FFA = free fatty acids, FS = free sterol, DAG =
 348 diacylglycerols, MAG = monoacylglycerols, PL = polar lipids.

349

350 Due to the complexity of the polar lipid fractions, these were further separated by
 351 2D TLC, see Fig. 2. Both lipid extracts contained similar classes of polar lipid but the
 352 intensity of the spots was higher for the chloroplast lipids. The polar lipids included the two
 353 major galactosyl diacylglycerides (MGDG and DGDG) and sulfolipid (SQDG). The spot near
 354 the origin was suspected to be TGDG but no standard was available to confirm. The major
 355 phospholipids in spinach were also spotted, such as PC and PG.



356

357 **Fig. 2.** Polar lipids separated on 2D TLC plate. (A): from spinach leaf; (B) from spinach
 358 chloroplast. Abbreviation denote: MGDG = monogalactosyl diacylglycerol, DGDG =
 359 digalactosyl diacylglycerol, SQDG = sulfoquinovosyl diacylglycerol, TGDG = trigalactosyl
 360 diacylglycerol, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PG =
 361 phosphatidylglycerol.

362

363 The concentration of each polar lipid class was determined by GC and the results
 364 are shown in Table 2. The chloroplast-rich fraction was highly concentrated in glycolipids
 365 with its amount double compared to that of phospholipids, while the lipid extracted directly
 366 from the leaf tissue possessed an equal amount of phospholipids and glycolipids. The results
 367 also confirmed the abundance of MGDG and DGDG in the spinach leaf and chloroplast. The
 368 ratio of phospholipids to glycolipids was comparable to that reported by Dörmann and
 369 Benning (2002), where approximately 70 % glycolipids content was detected. The
 370 phospholipids ratio was slightly higher in leaf than in the chloroplast and this was similar to
 371 the results reported by Wintermans (1960). Besides, not taking TGDG into account, the
 372 results obtained for spinach leaf lipid in this study was also comparable to those reported by
 373 Yunoki et al. (2009).

374 **Table 2**

375 Polar lipids compositions of spinach leaf and chloroplasts (mg/ g lipids)

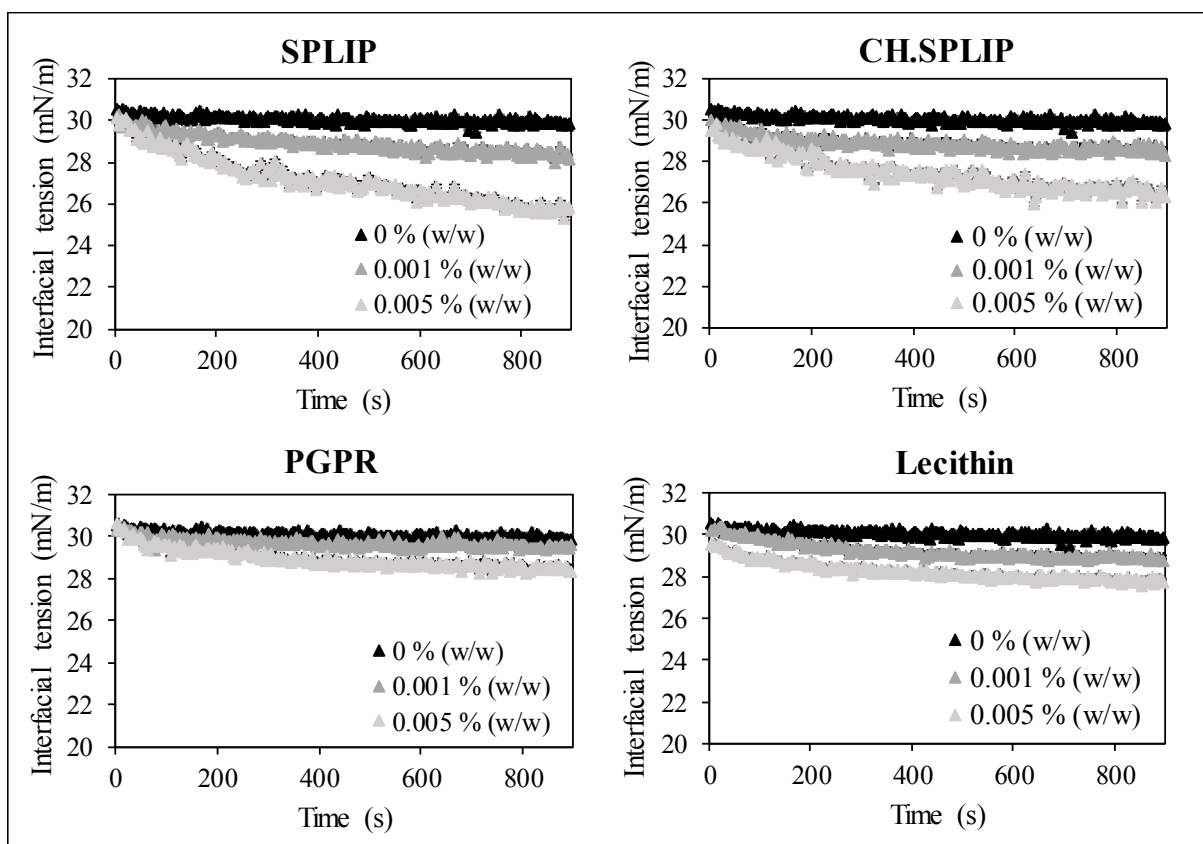
16:0 (palmitic)	6.8	12.5	0.7	0.6	4.3	5.8	24.1	15.0	41.6	45.6
16:1 (palmitoleic)	3.8	3.0	0.3	0.1	1.1	0.2	25.2	8.3	3.4	0.6
16:2 (polyenoic)	0.1	0.1	-	0.1	0.2	0.2	6.7	2.0	0.7	0.7
17:0 (margaric)	-	-	-	-	0.1	0.1	-	-	-	-
16:3 (n-3) (hexadecatrienoic)	11.1	0.8	18.6	20.6	3.3	3.3	-	-	0.6	1.0
18:0 (stearic)	0.4	0.6	0.3	0.2	0.8	1.1	13.9	16.3	2.5	2.3
18:1 (n-9) (elaidic)	1.0	2.7	-	0.1	0.4	0.9	2.9	4.4	2.9	0.6
18:1 (n-7) (vaccenic)	0.7	1.0	0.4	0.4	1.6	1.4	-	-	0.5	0.6
18:2 (n-6) (linoleic)	4.6	11.3	0.9	0.9	1.1	2.0	3.3	2.1	5.3	3.2
18:3 (n-3) (α - linolenic)	70.7	58.2	78.7	76.3	86.1	83.2	-	3.1	40.3	42.4
20:0 (arachidic)	0.1	0.4	-	0.1	0.2	0.2	4.4	21.3	0.5	0.5
20:1 (gondoic)	0.1	0.4	-	-	-	-	-	-	-	-
20:3 (n-3) (mead)	0.2	0.1	-	0.1	0.3	0.4	-	-	-	-
22:0 (behenic)	0.1	0.4	-	0.1	0.2	0.2	4.3	20.9	0.5	0.5
22:1 (erucic)	-	-	-	0.2	-	-	-	-	-	-
24:0 (lignoceric)	0.2	0.9	-	0.2	0.3	0.8	11.1	4.6	0.6	1.1

394

395 3.4. Interfacial tension at oil/water interface

396 The surface activity of the lipid extracts was assessed by measuring interfacial
397 tension at the oil/water interface at 20 °C and the results are reported in Fig. 3 alongside
398 reference data for 0 % added surfactant in the oil phase. As expected, the reference data
399 showed no time dependency, while the interfacial tension at the surfactant-laden interfaces
400 decreased initially followed by asymptotically approaching a constant value at times longer
401 than 600 s after generation of the interface. The only exception is the interfacial pair of 0.005
402 % SPLIP in oil/water for which equilibrium was approached later, around 900 s after
403 generation of the interface. Attainment of equilibrium was taken as a change of less than 1
404 mN/m in interfacial tension over at least 100 s measurement time. As expected, the data
405 tended to lower values at higher surfactant concentration. Equilibrium interfacial tension
406 values are reported in Table 4; the value for the pure oil/water interface was calculated by
407 averaging the data of the full 900 s of measurement and the other data were obtained by
408 averaging the value at 900 s for three replicate measurements. The interfacial tension at the
409 pure oil/water interface was 30.1 ± 0.2 mN/m and comparable to previously reported values

410 (Gaonkar & Borwankar, 1991; Gülseren & Corredig, 2012). This value was statistically
 411 significantly lower in the presence of 0.005 % (w/w) of either spinach lipid ($p < 0.05$). No
 412 further significant differences were observed as the concentration of surfactant was kept very
 413 low to allow sufficient optical contrast between the water droplet and the deep green oil
 414 phase containing spinach lipid. Nonetheless, the interfacial tension data gave evidence for the
 415 surface activity of the spinach lipid extracts and thus one would expect these to be
 416 functionally active as rheology modifiers in sugar/oil suspensions.



417
 418 **Fig. 3.** Surface tension at the water-oil interface of added surfactant either with leaf lipid,
 419 chloroplast lipid, lecithin or PGPR. Data plotted as means \pm 1 for standard deviation for $n=3$.

420

421 **Table 4**

422 Interfacial tension at the oil/water interface (20°C) in presence of spinach lipid extract from
 423 leaf or chloroplast, PGPR or lecithin (mean value recorded at 900 s). Different subscript
 424 letters indicate statistically significant difference in the data ($p < 0.05$).

Oil sample	Interfacial tension (mN.m⁻¹)
Control	30.0 ± 0.1 ^a
SPLIP 0.001 %	28.4 ± 0.4 ^{abc}
SPLIP 0.005 %	25.8 ± 1.0 ^c
CH.SPLIP 0.001 %	28.6 ± 0.9 ^{abc}
CH.SPLIP 0.005 %	26.6 ± 1.2 ^{bc}
PGPR 0.001 %	29.5 ± 1.5 ^{ab}
PGPR 0.005 %	28.7 ± 1.1 ^{abc}
LEC 0.001 %	28.9 ± 1.1 ^{ab}
LEC 0.005 %	27.8 ± 1.5 ^{abc}

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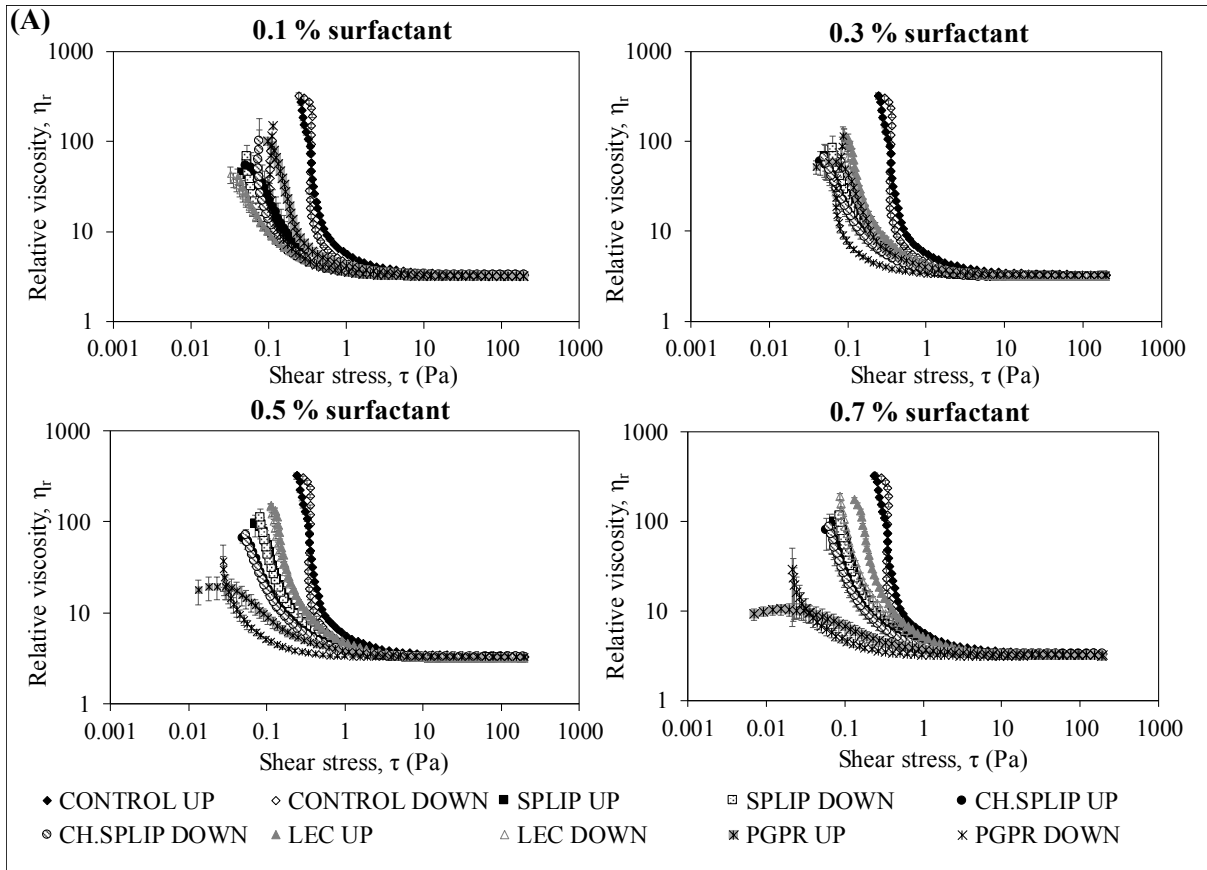
426 3.5. The effect of spinach lipids, PGPR and lecithin on the viscosity profile of sugar/oil
427 suspensions

428 The rheology modifying properties of the two natural spinach lipid extracts were
429 evaluated by comparing the shear rheological behavior of sugar/oil suspensions to those
430 prepared with PGPR and lecithin. The results are reported on the basis of the viscosity
431 profiles as well as the IOC chocolate rheology parameters recommended for use in industry
432 (International Office of Cocoa, 2000), and then discussed by suggesting a mechanistic model
433 for the rheology modifying properties of the spinach lipids. The apparent viscosity data
434 acquired by analyzing the sugar/oil suspensions with a sugar volume fraction of 0.28, 0.33 or
435 0.37 are presented in Fig. 4 – 6 as relative viscosity. At different sugar volume fraction, the
436 surfactant systems were applied between 0 – 0.7 % (w/w). Data are shown as a function of
437 shear stress to evaluate whether the natural lipid extracts would assume the functionality of
438 PGPR in oil-based suspensions, applied to modify rheology at low shear region.

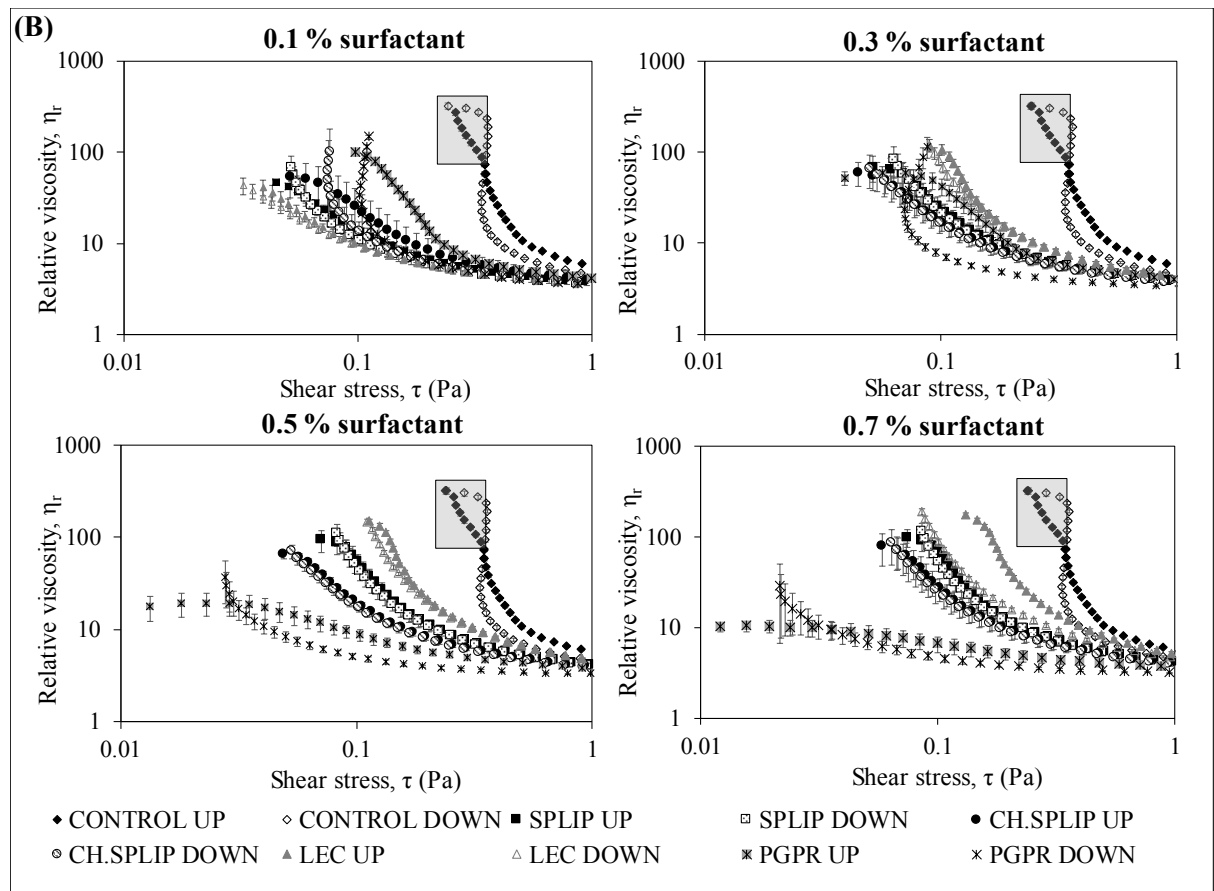
439 As a first observation, irrespective of surfactant system and concentration, the
440 sugar/oil suspensions showed a shear thinning behavior and a transition to a Newtonian
441 plateau at high shear stress. Referring to the increasing shear ramp data, and in accordance
442 with literature (Taylor, Van Damme, Johns, Routh, & Wilson, 2009), the minimum stress
443 required for the onset of flow is termed critical stress in the following discussion – to

444 distinguish from the yield stress definition as put forward by the IOC. However, the
445 increasing shear ramp data of a number of samples revealed initial elastic behavior prior to
446 transitioning to liquid-like behavior signified by the onset of the smooth sharply decreasing
447 apparent viscosity data trace. Where solid-like flow regions were observed, they are
448 highlighted in Fig. 4(B), 5(B) and 6(B). The final stress value of this region, corresponding to
449 the first stress value signifying liquid-like flow behavior, was taken as the critical stress.
450 Otherwise, the first stress value recorded was taken as the critical stress. However, some
451 initial data was clearly influenced by the fact that a pre-shear was applied (10 s^{-1} for 50 s), as
452 the data recorded at the initial shear stress values were lower than the data at higher shear. In
453 this instance, the first data point showing a decrease in apparent viscosity after this pre-shear
454 affected area was taken as the critical stress value, such as for the highest volume fraction
455 suspension containing 0.7 % (w/w) PGPR (Fig. 6).

456 At a sugar volume fraction of 0.28, the critical stress values ranged from 0.04 to 0.1
457 Pa at 0.1 % addition, in the order of lecithin (0.04 Pa), SPLIP (0.04 Pa), CH.SPLIP (0.05 Pa)
458 and PGPR (0.1 Pa). The data were comparable to each other at concentration more than 0.3
459 %, except that lecithin had an increased critical stress value, approaching 0.1 Pa. There was
460 also an increasing trend of apparent viscosity when the lecithin concentration was increased
461 from 0.1 % to 0.7 %. At a surfactant addition of 0.5 % and 0.7 %, PGPR imparted a
462 significant reduction in the critical stress and apparent viscosity, with values close to 0.05 Pa
463 in the shear thinning region and approaching a Newtonian plateau. The value recorded for
464 SPLIP and CH.SPLIP remained below 0.1 Pa while the value recorded for lecithin continued
465 to increase passing 0.1 Pa.



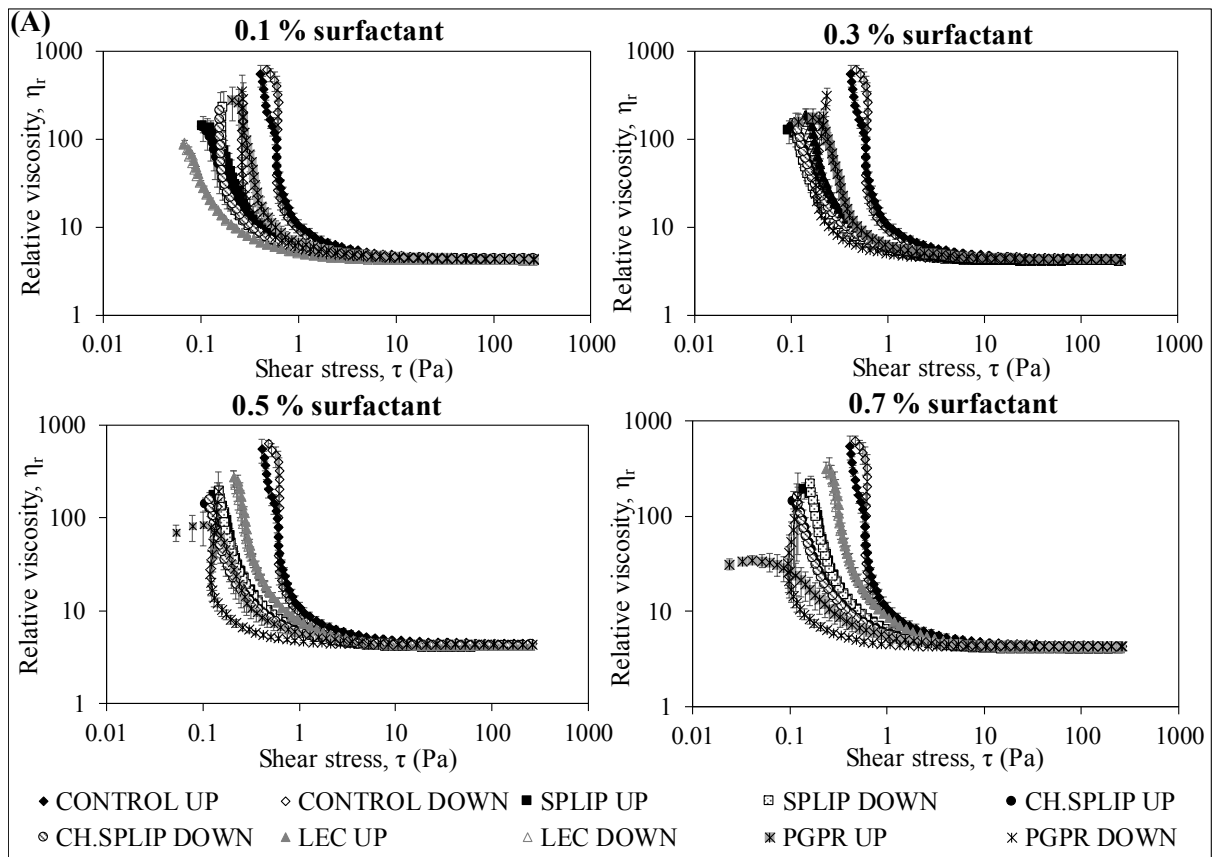
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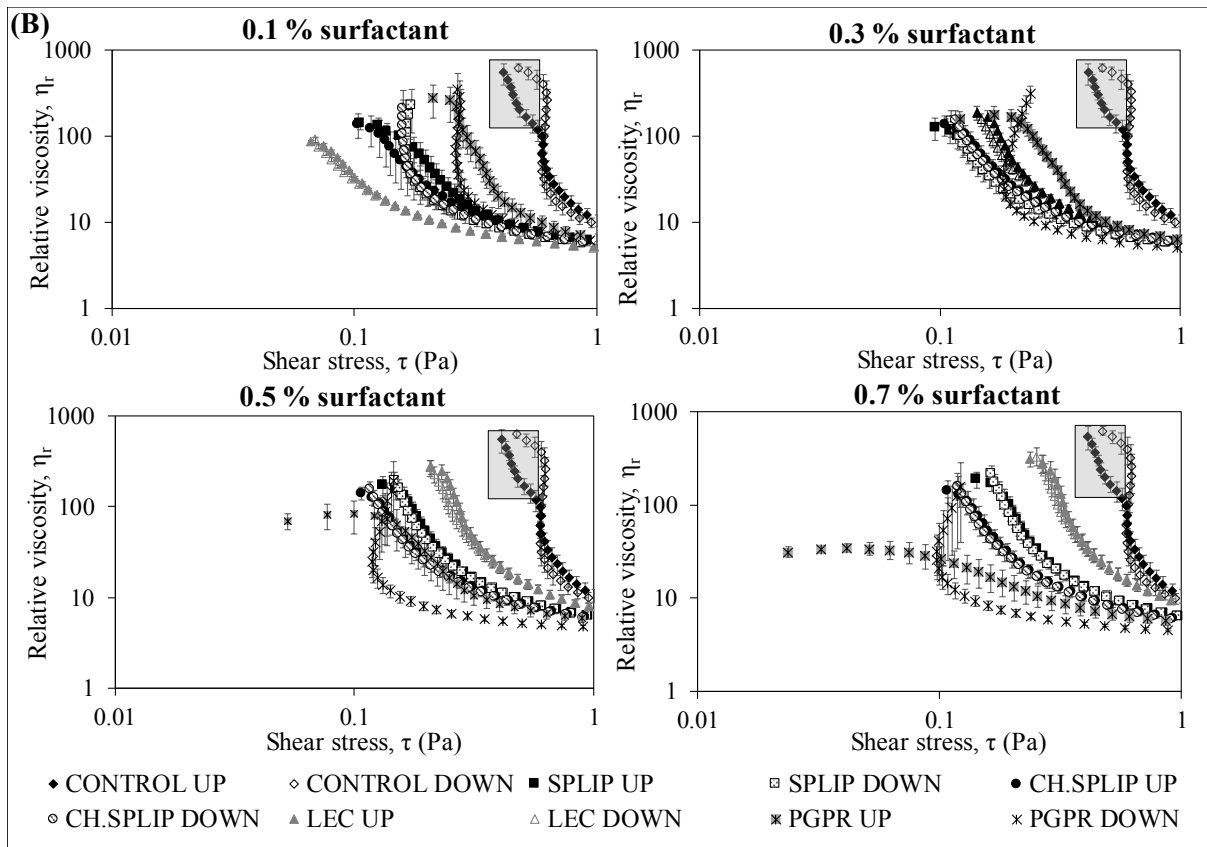
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468 **Fig. 4.** (A) Relative viscosity curves of sugar/oil suspensions at sugar volume fraction of 0.28
 469 as affected by the concentration of surfactant (mean \pm 1.0, n=4). (B) Low shear region data to
 470 aid yield stress discussion. Data acquired during shear rate increase and decrease respectively
 471 are labeled “UP” and “DOWN”. The greyed highlighted areas signify the transition from
 472 solid-like to liquid-like behavior.

473



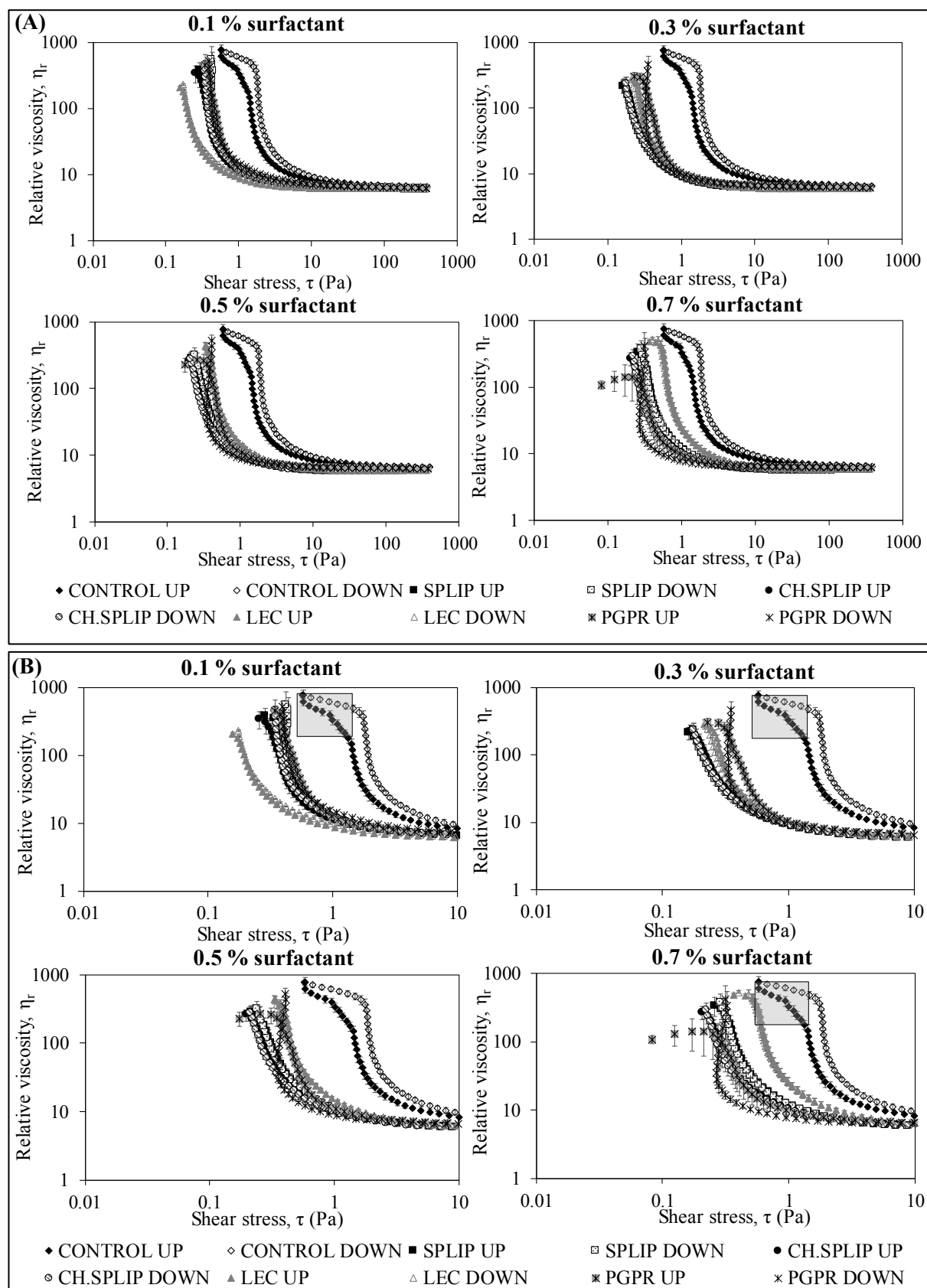
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476 **Fig. 5.** (A) Relative viscosity curves of sugar/oil suspensions at sugar volume fraction of 0.33
 477 as affected by the concentration of surfactant (mean \pm 1.0, n=4). (B) Low shear region data to
 478 aid yield stress discussion. Data acquired during shear rate increase and decrease respectively
 479 are labeled “UP” and “DOWN”. The greyed highlighted areas signify the transition from
 480 solid-like to liquid-like behavior.

481



482

483 **Fig. 6.** (A) Relative viscosity curves of sugar/oil suspensions at sugar volume fraction of 0.37484 as affected by the concentration of surfactant (mean \pm 1.0, n=4). (B) Low shear region data to

485 aid yield stress discussion. Data acquired during shear rate increase and decrease respectively
486 are labeled “UP” and “DOWN”. The greyed highlighted areas signify the transition from
487 solid-like to liquid-like behavior.

488

489 Comparing the data traces acquired upon increasing shear (“UP”) versus decreasing
490 shear (“DOWN”), it is evident that the control suspension and the suspensions containing
491 more than 0.1 % of PGPR were thixotropic. The decreasing shear ramp data at low shear
492 stress (0.05 Pa – 0.5 Pa) was below the values of the increasing shear ramp. The other
493 surfactant containing systems were not thixotropic. At even lower shear stresses (0.01 - 1 Pa,
494 depending on the sugar volume fraction), there was a point where the stress value of the
495 decreasing ramp remained constant, while the apparent viscosity value increased. By that
496 point, the sugar particles would strongly interact reducing the ability of the suspension to
497 flow, thus increasing its apparent viscosity.

498 In samples with a sugar volume fraction of 0.33, the critical stress value of the
499 suspensions had increased. At a surfactant addition of 0.1 %, the critical stress was between
500 0.07 Pa and 0.25 Pa, starting with lecithin as the lowest followed by the spinach lipids and
501 PGPR. As the case of 0.28 sugar volume fraction, lecithin also showed the lowest critical
502 stress value while PGPR showed the highest. At a surfactant addition of 0.3 %, similar trends
503 were seen with the critical stress value being around 0.10 – 0.20 Pa. At a surfactant addition
504 of 0.5 % and 0.7 %, the critical stress shown by PGPR was reduced to about 0.13 Pa and 0.05
505 Pa, respectively, with the apparent viscosity data in the shear thinning region approaching a
506 plateau. The values for the SPLIP and CH.SPLIP systems remained constant at about 0.11 to
507 0.14 Pa for both of these higher concentrations.

508 Similar observations were seen for the suspensions with the highest sugar volume
509 fraction (0.37). Lecithin showed the lowest critical stress value (around 0.2 Pa) at 0.1 %

510 addition while the other surfactants imparted a critical stress of around 0.30 - 0.35 Pa. At 0.3
511 % addition, all suspensions containing surfactant appeared to have a similar value of critical
512 stress, which was around 0.18 – 0.20 Pa. At the two higher surfactant concentrations (0.5 %
513 and 0.7 %), the critical stress of the suspension containing lecithin increased to 0.34 Pa and
514 0.52 Pa, respectively. As was mentioned before, the initial data for PGPR at 0.5 % and 0.7 %
515 had been influenced by the pre-shear sequence (10 s^{-1} for 50 s) during which a higher shear
516 than recorded as the first data point (0.01 s^{-1}) was applied. As a result, the first data point at
517 0.01 s^{-1} showed a lower apparent viscosity value than the subsequent data acquired at higher
518 shear. Therefore, the critical stress value for PGPR was taken where the data started to
519 decrease in apparent viscosity after that pre-shear affected area. This was at 0.30 Pa and 0.21
520 Pa for 0.5 % and 0.7 % addition, respectively. Systems containing SPLIP and CH.SPLIP
521 maintained a critical stress value of 0.20 - 0.25 Pa at the two higher concentrations of
522 surfactant.

523 Generally, all surfactants significantly affected the viscosity profile of the sugar/oil
524 suspensions, with lecithin showing the highest reduction in apparent viscosity at the lowest
525 concentration applied (0.1 %). The other surfactants only showed a comparable effect with
526 lecithin at 0.3 %. Increasing concentration to more than 0.3 % showed no further effect for
527 spinach lipids but it was different for lecithin and PGPR. In the case of lecithin, the critical
528 stress of the suspensions increased while in the case of PGPR, it was significantly lower at
529 addition of 0.5 % and 0.7 %. To compare the effect of the type and concentration of
530 surfactant at different sugar volume fractions in terms of significant difference in the mean
531 value of apparent viscosity and yield stress of suspensions, an evaluation following the
532 recommendation by the International Office of Cocoa (IOC) (2000) was carried out.

533

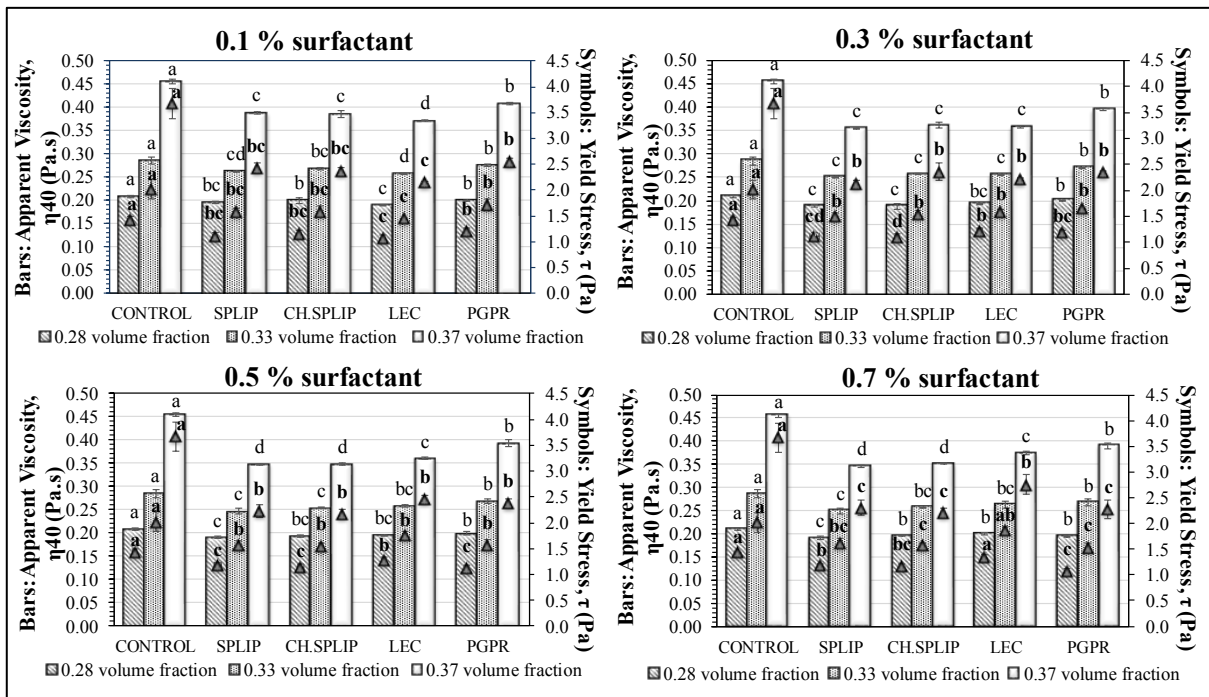
534 3.6. IOC parameters

535 As recommended by the IOC (2000), the apparent viscosity at shear rate 40 s^{-1} (η_{40})
536 and the yield stress (τ) at shear rate 5 s^{-1} were extracted from the shear rheological data. The
537 results are shown in Fig. 7. It can be seen that the addition of any surfactant into the sugar/oil
538 systems caused a significant reduction ($p < 0.05$) in the apparent viscosity and the yield stress
539 of the suspensions irrespective of the sugar volume fraction.

540 PGPR had the weakest apparent viscosity-reducing effect of all surfactants included
541 in this study, at all concentrations and for all three sugar phase volumes. The same behavior
542 in comparison to lecithin has previously been reported (Rector, 2000; Schantz & Rohm,
543 2005). In terms of apparent viscosity reduction, the two spinach lipid extracts performed in a
544 similar way to lecithin, or slightly better. There were no statistically significant differences (p
545 > 0.05) between the η_{40} values of the two spinach lipid extracts at the same surfactant
546 concentration and sugar phase volume.

547 The poor performance of PGPR as apparent viscosity reducing agent was not
548 surprising, as PGPR is applied to chocolate to affect yield stress. However, the results in
549 terms of yield stress reduction did not necessarily suggest that PGPR outperformed the other
550 surfactant systems as may have been expected. In fact, the only statistically significant
551 differences ($p < 0.05$) in yield stress between the systems containing surfactant at the same
552 added concentration was lecithin lowering the yield stress further than PGPR at 0.1 % for all
553 three sugar phase volumes. Comparing the performance of surfactant at the lowest sugar
554 volume fraction in terms of yield stress, CH.SPLIP lowered the yield stress further than
555 lecithin and PGPR at addition of 0.3 % and lecithin was not as effective as the other three
556 surfactant systems at 0.5 % as well as 0.7 %. PGPR was more effective than SPLIP and
557 lecithin at 0.7 % for all sugar volume fractions. At higher sugar volume fraction (0.33 and
558 0.37), CH.SPLIP performed comparably with PGPR in terms of yield stress at concentrations
559 of 0.3 % and above, while the effectiveness in lowering the apparent viscosity was more than

560 lecithin at 0.5 % and 0.7 %. Lecithin showed a reverse effect at high concentration,
 561 increasing the yield stress and apparent viscosity value, as described before by Schantz and
 562 Rohm (2005) and Beckett (2008). The reason for PGPR not standing out as much as
 563 expected, at least in comparison to lecithin, may be due to the relatively low particle phase
 564 volume included in this study, compared to real chocolate. As aforementioned, chocolate is
 565 formulated at particle phase volumes of up to 0.75. The lower values were selected here to
 566 circumvent difficulties in reproducible preparation of the suspensions and occurrence of
 567 measurement artifacts in the rheometer, in particular slip, may have compromised the value
 568 of the data in view to reformulating chocolate.



569

570 **Fig. 7.** Effect of spinach leaf lipid and chloroplast lipid in comparison to lecithin and PGPR
 571 on the viscosity (40 s^{-1}) and yield stress (5 s^{-1}) of sugar/oil suspensions. Letters indicate
 572 statistically significant difference ($p < 0.05$) between samples of same volume fraction. Bold
 573 letters refer to yield stress.

574

575 3.7. Behavior of surfactants at the interface of sugar and oil

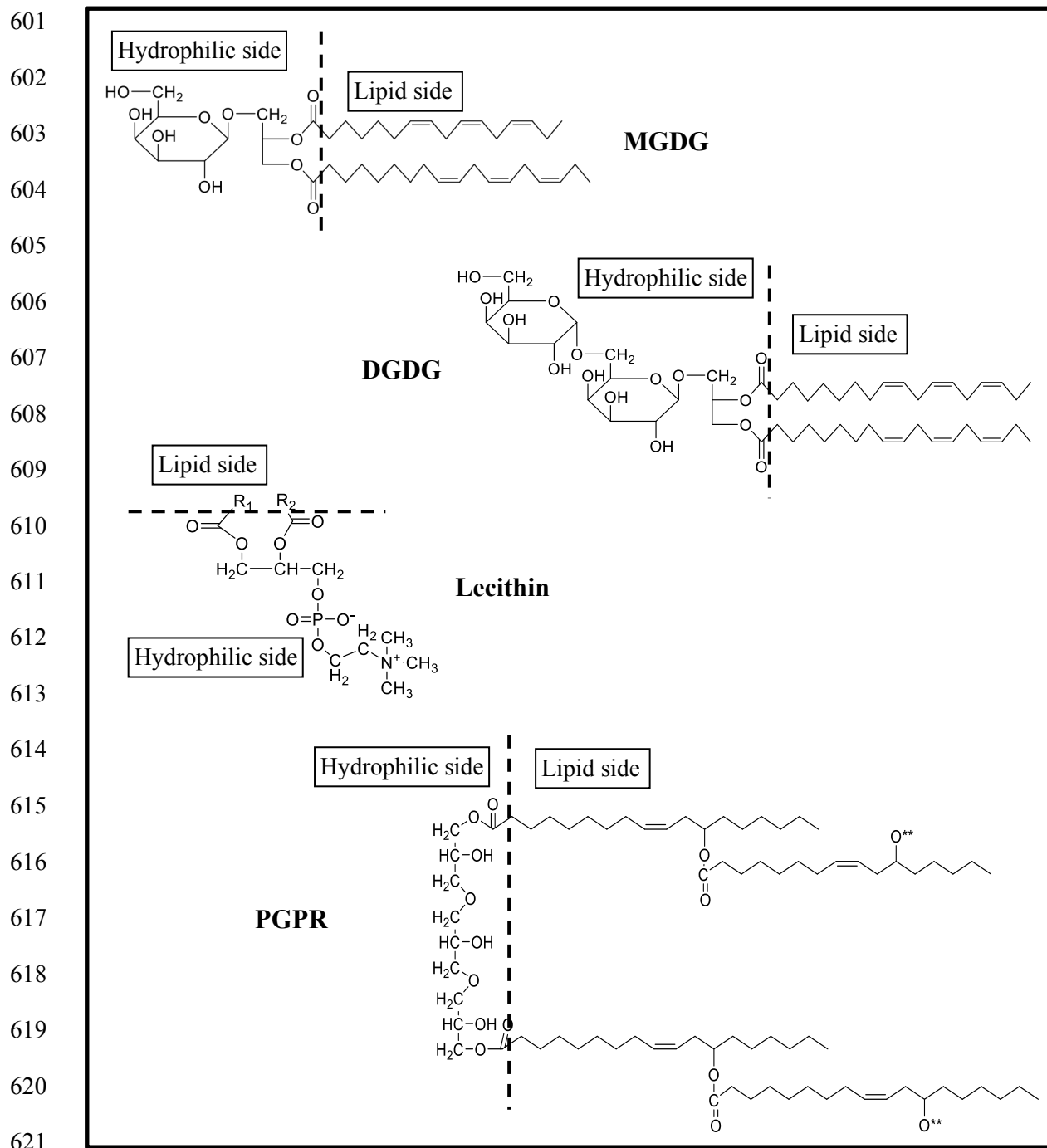
576 The behavior of lecithin at the interface of sugar and oil medium phase in modifying
577 the flow properties of oil based suspensions was described as a head-tail emulsifier (Beckett,
578 2008; Middendorf, Juadjur, Bindrich, & Mischnick, 2015). PGPR on the other hand was
579 reported to not follow the principle of a head-tail emulsifier like lecithin (Ziegler, Garbolino,
580 & Coupland, 2003). In the study by Middendorf et al. (2015), PGPR was seen to interact with
581 CB immobilized on the surface of sucrose forming pillow-like deposits between the
582 individual sucrose particles, thereby separating these by steric hindrance. The interaction of
583 the immobilized CB and PGPR caused a space (depletion area) which needed to be filled by
584 the CB from the bulk, thus enhancing the amount of immobilized fat instead of reducing
585 (Middendorf et al., 2015). As a result, PGPR does not affect apparent viscosity significantly.
586 The chemical structure of lecithin and PGPR are shown in Fig. 8.

587 Spinach lipids are a complex mixture of MGDG, DGDG, SQDG, PG and some PC
588 as the polar lipids (Mazliak, 1977). Due to the different molecular structure among these
589 lipids, it is difficult to understand the behavior of spinach lipids at the interface of sugar and
590 oil. MGDG has one galactose in its structure where it appears to have smaller head group
591 than DGDG which has two molecules of galactose (Fig. 8). With the bulk lipophilic tails,
592 MGDG molecules aggregate in the form of inverted rod like structure; not forming bilayers
593 but adopting the hexagonal-II (HII) phase with the polar head group facing toward the center
594 of the rod in water (Dörmann, 2013). On the other hand, DGDG with the larger size of polar
595 head group adopts a cylindrical shape which in turn forms bilayers in pure water (Dörmann,
596 2013). SQDG and PG also adopt a cylindrical shape and form bilayers when dispersed in
597 pure water (Kobayashi, 2016).

598

599

600



622 **Fig. 8.** Chemical structure of MGDG, DGDG, lecithin and PGPR. R₁ and R₂ are the fatty
 623 acids residues. The ** on the PGPR structure denotes polyricinoleic acid chains.

624

625 The anionic SQDG and PG have been demonstrated to play an important role in
 626 maintaining the bilayer structure of neutral lipids in studies where screening out these lipids
 627 have resulted in the fusion of the single-shell vesicles and remaining lipids formed larger

628 aggregates (Gounaris, Sen, Brain, Quinn, & Williams, 1983). The non-bilayer phase of
629 MGDG however, is understood to be a thermotropic mesophase where, at relatively low
630 temperatures, MGDG forms a bilayer structure and on heating it can transform into a non-
631 bilayer structure in excess water (Quinn, 2012). In this study, the process of extracting
632 spinach lipids involved heating at 80 °C but it is hard to predict the possible behavior of the
633 polar lipids when they are in a mixture, instead of their behavior individually. However, in
634 other studies, when lipids were extracted from heated spinach extract MGDG was reported to
635 have increased and the DGDG levels decreased compared to the unheated extract leading to a
636 suggestion that further reactions involving DGDG lead to the formation of MGDG (Cho, Lee,
637 Park, & Lee, 2001; Fricker et al., 1975). This non-bilayer structure should be beneficial to
638 give emulsifying effects on the sugar/oil suspension as the polar head of MGDG can adsorb
639 to the hydrophilic surface of sugar while the tails are facing the oil. DGDG and some PC in
640 the extract would also be expected to have a behavior like lecithin. Therefore, the apparent
641 viscosity lowering effect by spinach lipids are suggested to be due to the combined action of
642 MGDG, DGDG and PC. The negatively charged lipids of SQDG and PG (Fig. 9) are
643 important to avoiding particle aggregation thus helping to maintain the low yield stress of the
644 suspension and not showing the negative effect that lecithin gives when increasing the
645 concentration.

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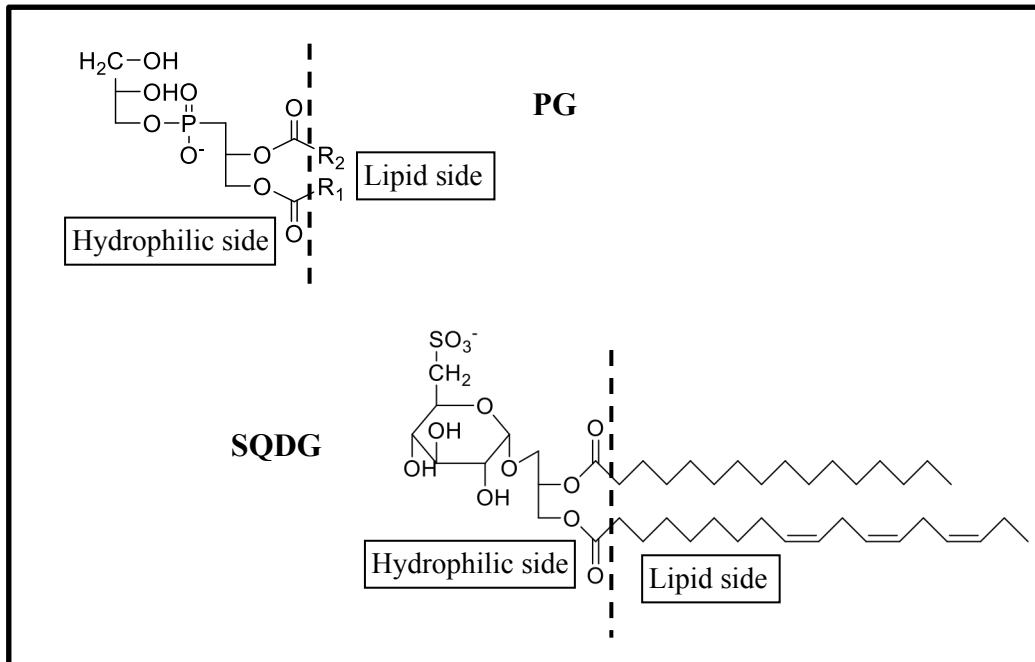
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665 **Fig. 9.** Chemical structure of PG and SQDG with the negative charge on the hydrophilic side.

666 R₁ and R₂ denote hydrocarbon chains of fatty acids.

667

668 4. Conclusions

669 The results presented in this study evidence the promising potential of lipids from
 670 spinach leaf and chloroplast for use as a rheology modifier in chocolate. The yield of lipid
 671 extracted from leaf was higher compared to chloroplast, albeit with a slightly different lipid
 672 composition. The glycolipids were the largest lipid group in the chloroplast fraction while the
 673 leaf showed an equal amount of phospholipids and glycolipids. Both lipid types were surface
 674 active at very low concentrations. The rheological study revealed a comparable effect of both
 675 spinach lipid types as a rheological modifier of a sugar/oil suspension. Therefore, even
 676 though glycolipids were more concentrated in chloroplasts, due to a comparable efficiency in
 677 modifying the rheological properties, the higher yield of lipids from leaf mean that it would
 678 be more beneficial to use the leaf extract so that the chloroplast isolation step can be omitted.
 679 The spinach lipids showed a better apparent viscosity reducing effect than lecithin, but at the

680 same time showed a comparable effect with PGPR in reducing the yield stress of the
681 suspensions. In conclusion, it appears worthwhile continuing to assess spinach lipid extract as
682 a clean label rheology modifier in chocolate, complementing the present data acquired on a
683 sugar/oil chocolate model.

684

685 **Acknowledgements**

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690

691 **References**

692

693 Afoakwa, E.O., Paterson, A., & Fowler, M. (2008). Effects of particle size distribution and
694 composition on rheological properties of dark chocolate. *European Food Research*
695 *and Technology*, 226(6), 1259-1268. <https://doi.org/10.1007/s00217-007-0652-6>

696 Afoakwa, E. O., Paterson, A., Fowler, M., & Vieira, J. (2009). Comparison of rheological
697 models for determining dark chocolate viscosity. *International Journal of Food*
698 *Science and Technology*, 44(1), 162-167. <https://doi.org/10.1111/j.1365->

699 [2621.2008.01710.x](https://doi.org/10.1111/j.1365-2621.2008.01710.x)

700 Allen, C. F., Good, P., Davis, H. F., Chisum, P., & Fowler, S. D. (1966). Methodology for the
701 separation o plant lipids and application to spinach leaf and chloroplast lamellae.
702 *Journal of the American Oil Chemists' Society*, 43(4), 223-231.

703 <https://doi.org/10.1007/BF02641091>

- 704 Andersson, M.X., & Dörmann, P. (2009). Chloroplast membrane lipid biosynthesis and
705 transport. *The Chloroplast* (pp. 125-158). Berlin, Heidelberg: Springer.
706 https://doi.org/10.1007/978-3-540-68696-5_4.
- 707 Arnold, G., Schuldt, S., Schneider, Y., Friedrichs, J., Babick, F., Werner, C., & Rohm, H.
708 (2013). The impact of lecithin on rheology, sedimentation and particle interactions in
709 oil-based dispersions. *Colloids and Surfaces a-Physicochemical and Engineering*
710 *Aspects*, 418, 147-156. <https://doi.org/10.1016/j.colsurfa.2012.11.006>
- 711 Beckett, S. T. (2008). *Science of Chocolate, 2nd Edition*. United Kingdom: RSC Publishing.
712 <https://doi.org/10.1039/9781847558053>.
- 713 Beckett, S. T. (2009). Chocolate Flow Properties. In S. T. Beckett (Ed.), *Industrial Chocolate*
714 *Manufacture And Use* (pp. 224-246). United Kingdom: Wiley-Blackwell.
715 <https://doi.org/10.1002/9781118923597.ch11>.
- 716 Benson, A. A. (1964). Plant membrane lipids. *Annual Review of Plant Physiology*, 15(1), 1-
717 16. <https://doi.org/10.1146/annurev.pp.15.060164.000245>
- 718 Cho, E., Lee, J., Park, K., & Lee, S. (2001). Effects of heat pretreatment on lipid and
719 pigments of freeze-dried spinach. *Journal of Food Science*, 66(8), 1074-1079.
720 <https://doi.org/10.1111/j.1365-2621.2001.tb16083.x>
- 721 Christiansen, K. (2014). PGPR, Polyglycerolpolyricinoleate, E476. *Emulsifiers in Food*
722 *Technology* (pp. 209-230): John Wiley & Sons, Ltd.
723 <https://doi.org/10.1002/9781118921265.ch9>.
- 724 Christie, W. W. (2012). The Lipid Library. *Preparation of lipid extracts from tissues*.
725 [http://lipidlibrary.aocs.org/lipid-analysis/selected-topics-in-the-analysis-of-](http://lipidlibrary.aocs.org/lipid-analysis/selected-topics-in-the-analysis-of-lipids/preparation-of-lipid-extracts-tissues)
726 [lipids/preparation-of-lipid-extracts-tissues](http://lipidlibrary.aocs.org/lipid-analysis/selected-topics-in-the-analysis-of-lipids/preparation-of-lipid-extracts-tissues) Accessed July 2015.

- 727 Doehlert, D. C., Moreau, R. A., Welti, R., Roth, M. R., & McMullen, M. S. (2010). Polar
728 Lipids from Oat Kernels. *Cereal Chemistry*, 87(5), 467-474.
729 <https://doi.org/10.1094/cchem-04-10-0060>
- 730 Doraiswamy, D., Mujumdar, A. N., Tsao, I., Beris, A. N., Danforth, S. C., & Metzner, A. B.
731 (1991). The Cox–Merz rule extended: a rheological model for concentrated
732 suspensions and other materials with a yield stress. *Journal of Rheology*, 35(4), 647-
733 685. <https://doi.org/10.1122/1.550184>
- 734 Dörmann, P. (2013). Galactolipids in plant membranes. *eLS*.
735 <https://doi.org/10.1002/9780470015902.a0020100.pub2>
- 736 Dörmann, P. & Benning, C. (2002). Galactolipids rule in seed plants. *Trends in Plant*
737 *Science*, 7(3), 112-118. [https://doi.org/10.1016/S1360-1385\(01\)02216-6](https://doi.org/10.1016/S1360-1385(01)02216-6)
- 738 Douce, R. (1974). Site of biosynthesis of galactolipids in spinach chloroplasts. *Science*,
739 183(4127), 852-853. <https://doi.org/10.1126/science.183.4127.852>
- 740 Douce, R., Holtz, R. B., & Benson, A. A. (1973). Isolation and properties of the envelope of
741 spinach chloroplasts. *Journal of Biological Chemistry*, 248(20), 7215-7222.
742 <https://www.jbc.org/content/248/20/7215.long> Accessed June 2018
- 743 Evans, R., Jee, M. H., Sander, N. H., Smith, I. H., & Gibson, R. K. (1991). United States
744 Patent No. Retrieved from
745 [https://patentimages.storage.googleapis.com/02/95/d6/0d06ef0e37d6a4/US5026548.p](https://patentimages.storage.googleapis.com/02/95/d6/0d06ef0e37d6a4/US5026548.pdf)
746 [df](https://patentimages.storage.googleapis.com/02/95/d6/0d06ef0e37d6a4/US5026548.pdf) Accessed July 2018.
- 747 Fishwick, M. J., & Wright, A. J. (1977). Comparison of methods for the extraction of plant
748 lipids. *Phytochemistry*, 16(10), 1507-1510. [https://doi.org/10.1016/0031-](https://doi.org/10.1016/0031-9422(77)84011-9)
749 [9422\(77\)84011-9](https://doi.org/10.1016/0031-9422(77)84011-9)

- 750 Folch, J., Lees, M. & Stanley, G. H. S. (1957). A simple method for the isolation and
751 purification of total lipids from animal tissues. *Journal of Biological Chemistry*,
752 226(1), 497-509. <http://www.jbc.org/content/226/1/497.full.pdf> Accessed July 2015.
- 753 Fricker, A, Duben, R, Heintze, K, Panlas, K, & Zohm, H. (1975). Influence of heat treatment
754 of spinach at temperatures up to 100 C on important constituents: Total lipids and
755 glycolipids. *Lebensm Wiss u Technol*, 8, 172-186.
- 756 Gaonkar, A. G., & Borwankar, R. P. (1991). Competitive adsorption of monoglycerides and
757 lecithin at the vegetable oil-water interface. *Colloids and Surfaces*, 59, 331-343.
758 [https://doi.org/http://dx.doi.org/10.1016/0166-6622\(91\)80256-N](https://doi.org/http://dx.doi.org/10.1016/0166-6622(91)80256-N)
- 759 Gedi, M. A., Briars, R., Yuseli, F., Zainol, N., Darwish, R., Salter, A. M., & Gray, D. A.
760 (2017). Component analysis of nutritionally rich chloroplasts: recovery from
761 conventional and unconventional green plant species. *Journal of Food Science and*
762 *Technology*, 54(9), 2746-2757. <https://doi.org/10.1007/s13197-017-2711-8>
- 763 Goncalves, E. V., & Lannes, S. C. D. (2010). Chocolate rheology. *Ciencia E Tecnologia De*
764 *Alimentos*, 30(4), 845-851. <https://doi.org/10.1590/s0101-20612010000400002>
- 765 Gounaris, K., Mannock, D. A., Sen, A., Brain, A. P. R., Williams, W. P., & Quinn, P. J.
766 (1983). Polyunsaturated fatty acyl residues of galactolipids are involved in the control
767 of bilayer/non-bilayer lipid transitions in higher plant chloroplasts. *Biochimica et*
768 *Biophysica Acta (BBA) - Biomembranes*, 732(1), 229-242.
769 [https://doi.org/http://dx.doi.org/10.1016/0005-2736\(83\)90207-9](https://doi.org/http://dx.doi.org/10.1016/0005-2736(83)90207-9)
- 770 Gounaris, K., Sen, A., Brain, A. P. R., Quinn, P. J., & Williams, W. P. (1983). The formation
771 of non-bilayer structures in total polar lipid extracts of chloroplast membranes.
772 *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 728(1), 129-139.
773 [https://doi.org/10.1016/0005-2736\(83\)90445-5](https://doi.org/10.1016/0005-2736(83)90445-5)

- 774 Gülseren, İ., & Corredig, M. (2012). Interactions at the interface between hydrophobic and
775 hydrophilic emulsifiers: Polyglycerol polyricinoleate (PGPR) and milk proteins,
776 studied by drop shape tensiometry. *Food Hydrocolloids*, 29(1), 193-198.
777 <https://doi.org/http://dx.doi.org/10.1016/j.foodhyd.2012.03.010>
- 778 International Office of Cocoa, IOC. (2000). Viscosity of Cocoa and Chocolate Products
779 *Analytical Methods 46* (Available from CAOBISCO, rue Defacqz 1, B-1000
780 Bruxelles, Belgium).
- 781 Jaime, L., Vazquez, E., Fornari, T., Lopez-Hazas, M. D., Garcia-Risco, M. R., Santoyo, S., &
782 Reglero, G. (2015). Extraction of functional ingredients from spinach (*Spinacia*
783 *oleracea* L.) using liquid solvent and supercritical CO₂ extraction. *Journal of the*
784 *Science of Food and Agriculture*, 95(4), 722-729. <https://doi.org/10.1002/jsfa.6788>
- 785 Kaimainen, M., Ahvenainen, S., Kaariste, M., Jarvenpaa, E., Kaasalainen, M., Salomaki, M.,
786 Salonen, J., & Huopalahti, R. (2012). Polar lipid fraction from oat (*Avena sativa*):
787 characterization and use as an o/w emulsifier. *European Food Research and*
788 *Technology*, 235(3), 507-515. <https://doi.org/10.1007/s00217-012-1780-1>
- 789 Kates, M., & Eberhardt, F. M. (1957). Isolation and fractionation of leaf phosphatides.
790 *Canadian Journal of Botany*, 35(6), 895-905. <https://doi.org/10.1139/b57-074>
- 791 Kobayashi, K. (2016). Role of membrane glycerolipids in photosynthesis, thylakoid
792 biogenesis and chloroplast development. *Journal of Plant Research*, 129(4), 565-580.
793 <https://doi.org/10.1007/s10265-016-0827-y>
- 794 Mazliak, P. (1977). Glyco- and Phospholipids of Biomembranes in Higher Plants. In M.
795 Tevini & H.K. Lichtenthaler (Eds.), *Lipids and Lipid Polymers in Higher Plants* (pp.
796 48-74). Berlin, Heidelberg: Springer Berlin Heidelberg. [https://doi.org/10.1007/978-](https://doi.org/10.1007/978-3-642-66632-2_3)
797 [3-642-66632-2_3](https://doi.org/10.1007/978-3-642-66632-2_3).

- 798 Melo, N., Tavares, R. M., Morais, F., Barroso, J. G., & Pais, M. S. S. (1995). Lipid
799 composition of thylakoid membranes from leaves and regreened spathes of
800 *Zantedeschia aethiopica*. *Phytochemistry*, *40*(5), 1367-1371.
801 [https://doi.org/10.1016/0031-9422\(95\)00506-3](https://doi.org/10.1016/0031-9422(95)00506-3)
- 802 Menke, W. (1938). Untersuchungen über das Protoplasma grüner Pflanzenzellen. I.
803 Isolierung von Chloroplasten aus Spinatblättern. *Hoppe-Seyler's Zeitschrift für*
804 *physiologische Chemie*, *257*(1), 43-48. <https://doi.org/10.1515/bchm2.1938.257.1.43>
- 805 Middendorf, D., Juadjur, A., Bindrich, U., & Mischnick, P. (2015). AFM approach to study
806 the function of PGPR's emulsifying properties in cocoa butter based suspensions.
807 *Food Structure*, *4*, 16-26. <https://doi.org/10.1016/j.foostr.2014.11.003>
- 808 Neufeld, Elizabeth F., & Hall, Clara W. (1964). Formation of galactolipids by chloroplasts.
809 *Biochemical and Biophysical Research Communications*, *14*(6), 503-508.
810 [https://doi.org/https://doi.org/10.1016/0006-291X\(64\)90259-1](https://doi.org/https://doi.org/10.1016/0006-291X(64)90259-1)
- 811 Nishimura, M., Graham, D., & Akazawa, T. (1976). Isolation of intact chloroplasts and other
812 cell organelles from spinach leaf protoplasts. *Plant Physiology*, *58*(3), 309-314.
813 <https://doi.org/10.1104/pp.58.3.309>
- 814 Osborn, S. (2015). Labelling relating to natural ingredients and additives. In P. Berryman
815 (Ed.), *Advances in Food and Beverage Labelling* (pp. 207-221): Woodhead
816 Publishing. <https://doi.org/10.1533/9781782420934.3.207>.
- 817 Östbring, K., Rayner, M., Albertsson, P.Å., & Erlanson-Albertsson, C. (2015). Heat-induced
818 aggregation of thylakoid membranes affect their interfacial properties. *Food &*
819 *Function*, *6*(4), 1310-1318. <https://doi.org/10.1039/C4FO01074D>
- 820 Quinn, P. J. (2012). Lipid–lipid interactions in bilayer membranes: Married couples and
821 casual liaisons. *Progress in Lipid Research*, *51*(3), 179-198.
822 <https://doi.org/http://dx.doi.org/10.1016/j.plipres.2012.01.001>

- 823 Rayner, M., Emek, S. C., Gustafssona, K., Erlanson-Albertsson, C., & Albertsson, P. Å.
824 (2011). A novel emulsifier from spinach with appetite regulation abilities. In G.
825 Saravacos, P. Taoukis, M. Krokida, V. Karathanos, H. Lazarides, N. Stoforos, C.
826 Tzia, & S. Yanniotis (Eds.), *11th International Congress on Engineering and Food*
827 (Vol. 1, pp. 1431-1438). Amsterdam: Elsevier Science Bv.
828 <https://doi.org/10.1016/j.profoo.2011.09.212>.
- 829 Rayner, M., Ljusberg, H., Emek, S. C., Sellman, E., Erlanson-Albertsson, C., & Albertsson,
830 P. Å. (2011). Chloroplast thylakoid membrane-stabilised emulsions. *Journal of the*
831 *Science of Food and Agriculture*, 91(2), 315-321. <https://doi.org/10.1002/jsfa.4187>
- 832 Rector, D. (2000). Chocolate-controlling the flow. *The Manufacturing Confectioner*, 80, 63 -
833 70. <http://www.gomc.com/firstpage/200005063.pdf> Accessed July 2018.
- 834 Sahasrabudhe, M. R. (1979). Lipid composition of oats (*Avena sativa* L.). *Journal of the*
835 *American Oil Chemists' Society*, 56(2), 80. <https://doi.org/10.1007/BF02914274>
- 836 Schantz, B., & Rohm, H. (2005). Influence of lecithin-PGPR blends on the rheological
837 properties of chocolate. *Lwt-Food Science and Technology*, 38(1), 41-45.
838 <https://doi.org/10.1016/j.lwt.2004.03.014>
- 839 Servais, C, Ranc, H, & Roberts, I. D. (2003). Determination of chocolate viscosity. *Journal*
840 *of Texture Studies*, 34(5-6), 467-497. [https://doi.org/10.1111/j.1745-](https://doi.org/10.1111/j.1745-4603.2003.tb01077.x)
841 [4603.2003.tb01077.x](https://doi.org/10.1111/j.1745-4603.2003.tb01077.x)
- 842 Siebertz, H. P., Heinz, E., Linscheid, M., Joyard, J., & Douce, R. (1979). Characterization of
843 lipids from chloroplast envelopes. *European Journal of Biochemistry*, 101(2), 429-
844 438. <https://doi.org/10.1111/j.1432-1033.1979.tb19736.x>
- 845 Taylor, J. E, Van Damme, I, Johns, M. L., Routh, A. F., & Wilson, D. I. (2009). Shear
846 rheology of molten crumb chocolate. *Journal of Food Science*, 74(2), E55-E61.
847 <https://doi.org/10.1111/j.1750-3841.2008.01041.x>

- 848 van Nieuwenhuyzen, W. (2010). Lecithin and Other Phospholipids. In M. Kjellin & I.
849 Johansson (Eds.), *Surfactants from Renewable Resources* (pp. 191 - 211). Great
850 Britain: A John Wiley and Sons, Ltd., Publication.
851 <https://doi.org/10.1002/9780470686607.ch10>.
- 852 Whitaker, B. D. (1986). Fatty-acid composition of polar lipids in fruit and leaf chloroplasts of
853 "16:3"- and "18:3"-plant species. *Planta*, *169*(3), 313-319.
854 <https://doi.org/10.1007/bf00392125>
- 855 Wintermans, J. F. G. M. (1960). Concentrations of phosphatides and glycolipids in leaves and
856 chloroplasts. *Biochimica Et Biophysica Acta*, *44*, 49-54.
857 [https://doi.org/http://dx.doi.org/10.1016/0006-3002\(60\)91521-3](https://doi.org/http://dx.doi.org/10.1016/0006-3002(60)91521-3)
- 858 Yao, L., Gerde, J. A., & Wang, T. (2012). Oil extraction from microalga *Nannochloropsis* sp.
859 with isopropyl alcohol. *Journal of the American Oil Chemists' Society*, *89*(12), 2279-
860 2287. <https://doi.org/10.1007/s11746-012-2124-9>
- 861 Youngs, V. L., Puskulcu, M., & Smith, R. R. (1977). Oat lipids. I. Composition and
862 distribution of lipid components in two oat cultivars. *Cereal Chemistry*, *54*, 803-812.
863 https://www.aaccnet.org/publications/cc/backissues/1977/Documents/chem54_803.pdf
864 [f](#) Accessed July 2018.
- 865 Yunoki, K., Sato, M., Seki, K., Ohkubo, T., Tanaka, Y., & Ohnishi, M. (2009). Simultaneous
866 quantification of plant glyceroglycolipids including sulfoquinovosyldiacylglycerol by
867 HPLC–ELSD with binary gradient elution. *Lipids*, *44*(1), 77-83.
868 <https://doi.org/10.1007/s11745-008-3248-4>
- 869 Ziegler, G. R., Garbolino, C., & Coupland, J. N. (2003). The influence of surfactants and
870 moisture on the colloidal and rheological properties of model chocolate dispersions.
871 Paper presented at the 3rd International Symposium on Food Rheology and Structure,
872 Zurich, Switzerland.

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