Spinach leaf and chloroplast lipid

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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 F_{254} 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 $KHCO_3$ 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 N_2 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 ºC 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 mm^2, 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 ºC 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
Volume (%)	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 ± 0.02 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 ± 0.04 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 aforenoted, 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 °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 ±

306   0.002 Pa.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 ± 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 (± 4.5) g/100 g dried spinach leaf and 24.0 (± 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 (± 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 (± 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
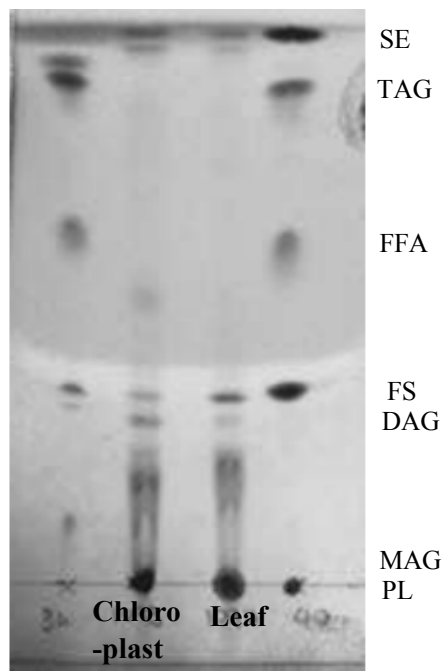
346   **Fig. 1.** Separation of lipid from spinach leaf and spinach chloroplast. Abbreviation denote:

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

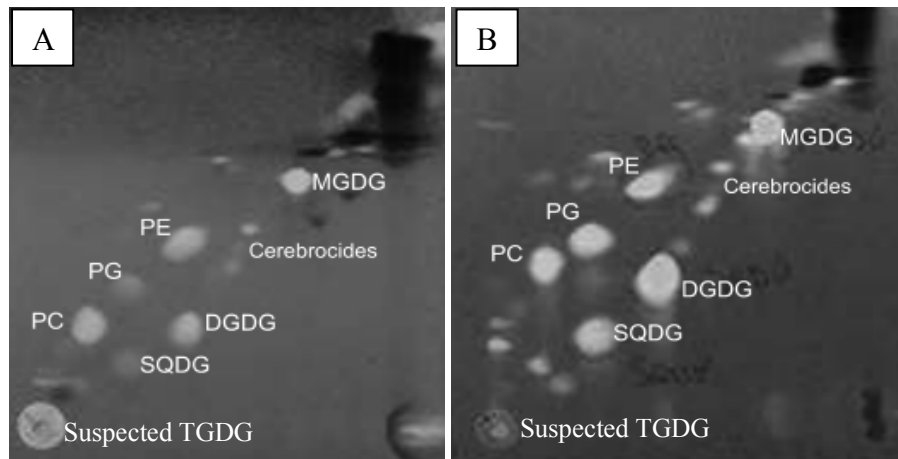355   phospholipids in spinach were also spotted, such as PC and PG.

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

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

Table 2

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

	Total phospho-lipids (PhL)	Total glycolipids (GL)	Ratio PhL:GL	Individual glycolipids				Ratio M:D:T:S
				MGDG (M)	DGDG (D)	TGDG (T)	SQDG (S)	
Chloroplast	181.8	418.9	30:70	202.4	124.2	53.2	39.1	48:30:13:9
Leaf	201.6	240.7	46:54	113.7	79.6	24.8	22.6	47:33:10:9

376

377   3.3.   Fatty acids composition

378   The fatty acids composition of the polar lipids from spinach chloroplast (CH) and

379 leaf (L) are tabulated in Table 3. Generally, the polar lipids were highly concentrated in the

380 two trienoic acids: hexadecatrienoic and α-linolenic acid. The amount of α-linolenic acid was

381 particularly high, accounting for 71 % and 58 % of the total lipids in chloroplast and leaf,

382 respectively. MGDG and DGDG were made up of more than 75 % α- linolenic acid, similar

383 to what has previously been reported (Gounaris, Mannock, et al., 1983; Melo, Tavares,

384 Morais, Barroso, & Pais, 1995). The high content of polyunsaturated fatty acids in

385 chloroplast lipid is very important as to maintain their biological function at low temperatures

386 (Andersson & Dörmann, 2009). The amount of hexadecatrienoic acid in MGDG was higher

387 than in other polar lipids, comparable to what has previously been reported for solanaceous

388 leaf (Whitaker, 1986). The presence of hexadecatrienoic (16:3) classifies spinach as a 16:3

389 plant, as this fatty acid is only present in certain plants (Andersson & Dörmann, 2009).

390 SQDG had an equal amount of 16:0 and 18:3 fatty acids as previously reported (Siebertz,

391 Heinz, Linscheid, Joyard, & Douce, 1979).

392 **Table 3**

393 Fatty acid composition of polar lipids from spinach leaf and chloroplasts (% total lipids)

Fatty acids	Total Polar Lipids		MGDG		DGDG		TGDG		SQDG	
	CH	L	CH	L	CH	L	CH	L	CH	L
14:0 (myristic)	0.1	0.1	-	0.1	0.1	0.2	4.2	2.0	0.5	0.7
15:0 (pentadecylic)	-	0.1	-	-	-	-	-	-	-	0.4

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.
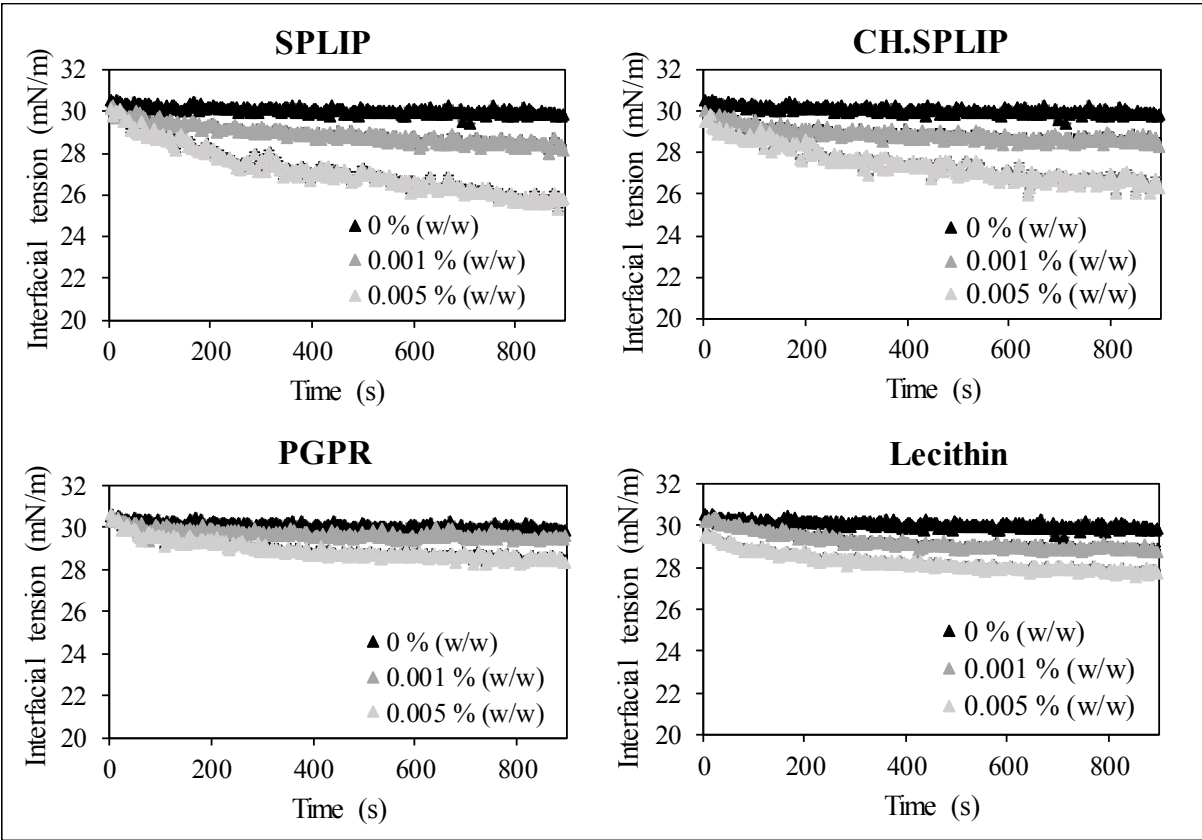
418 **Fig. 3.** Surface tension at the water-oil interface of added surfactant either with leaf lipid,

419 chloroplast lipid, lecithin or PGPR. Data plotted as means ± 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 0.00 5%	27.8 ± 1.5^{abc}

425

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.
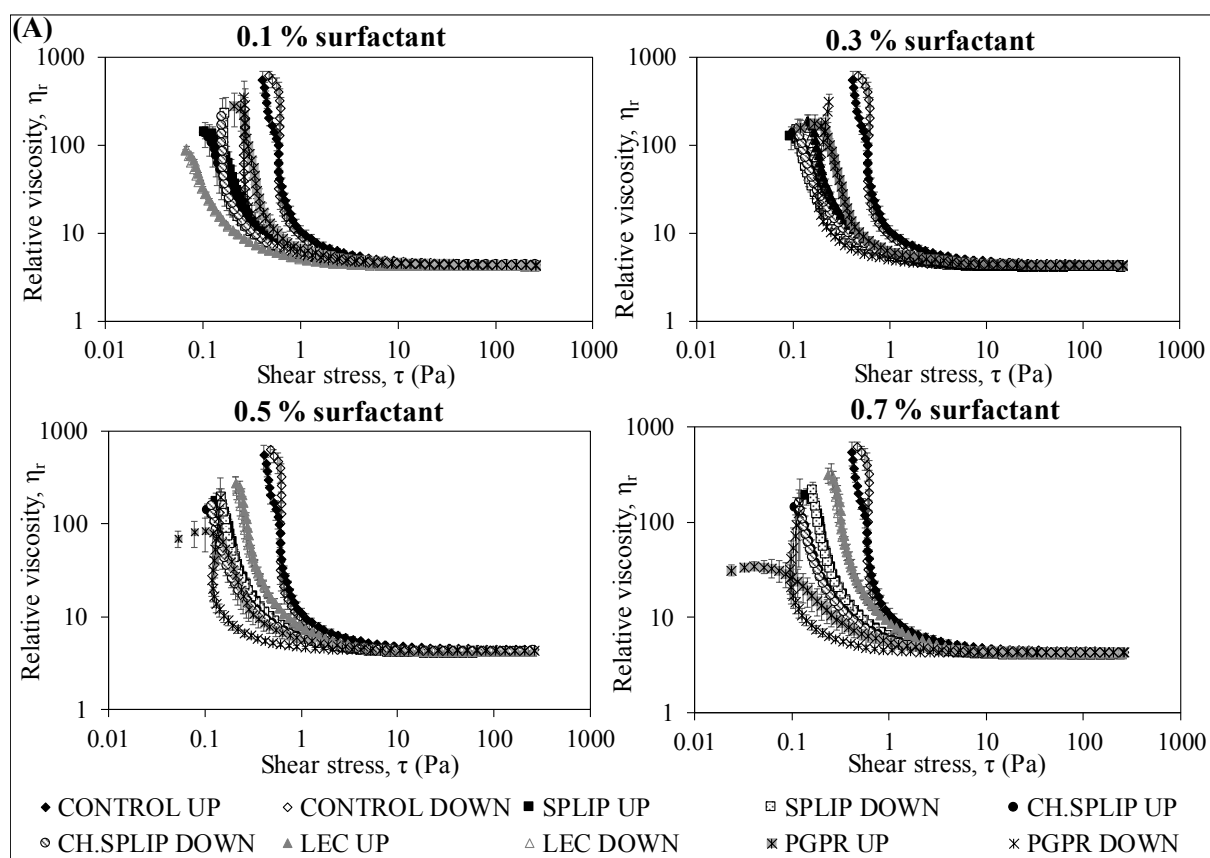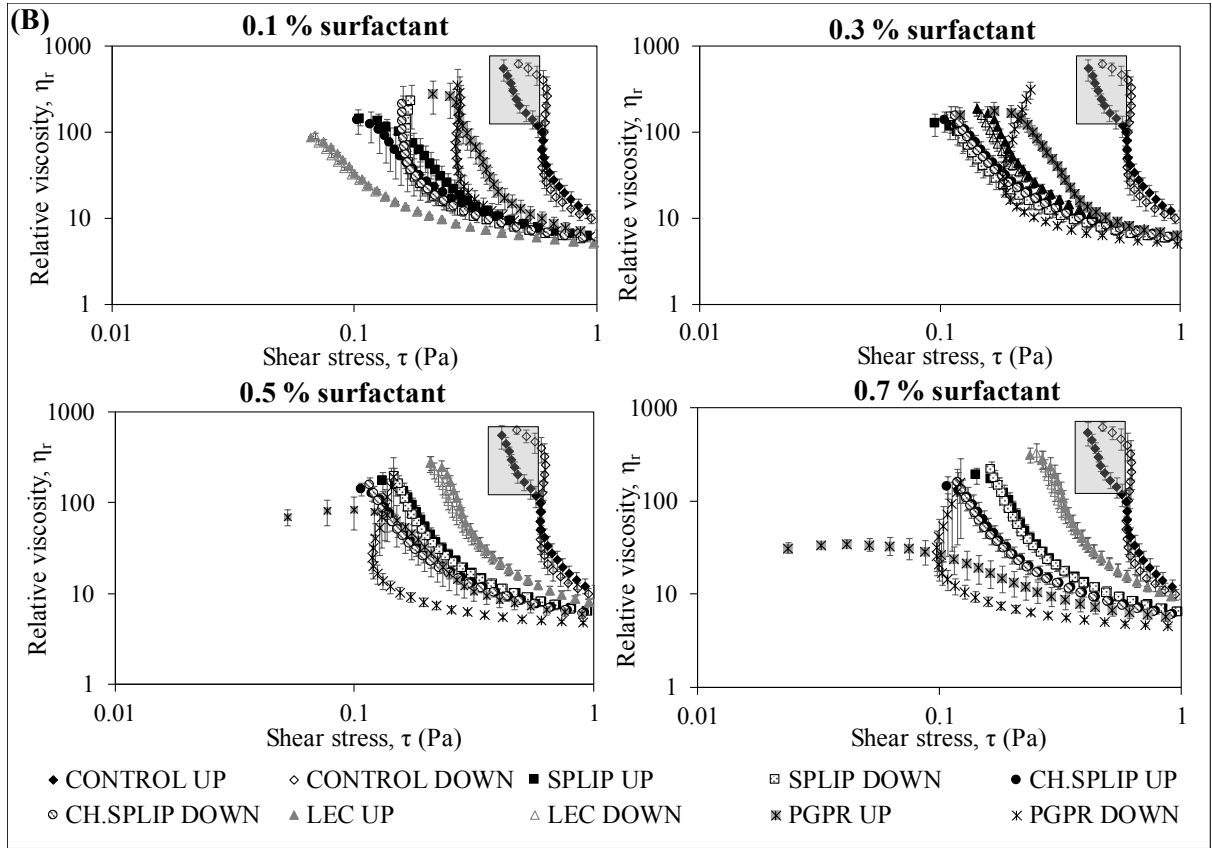
(A)

(B)

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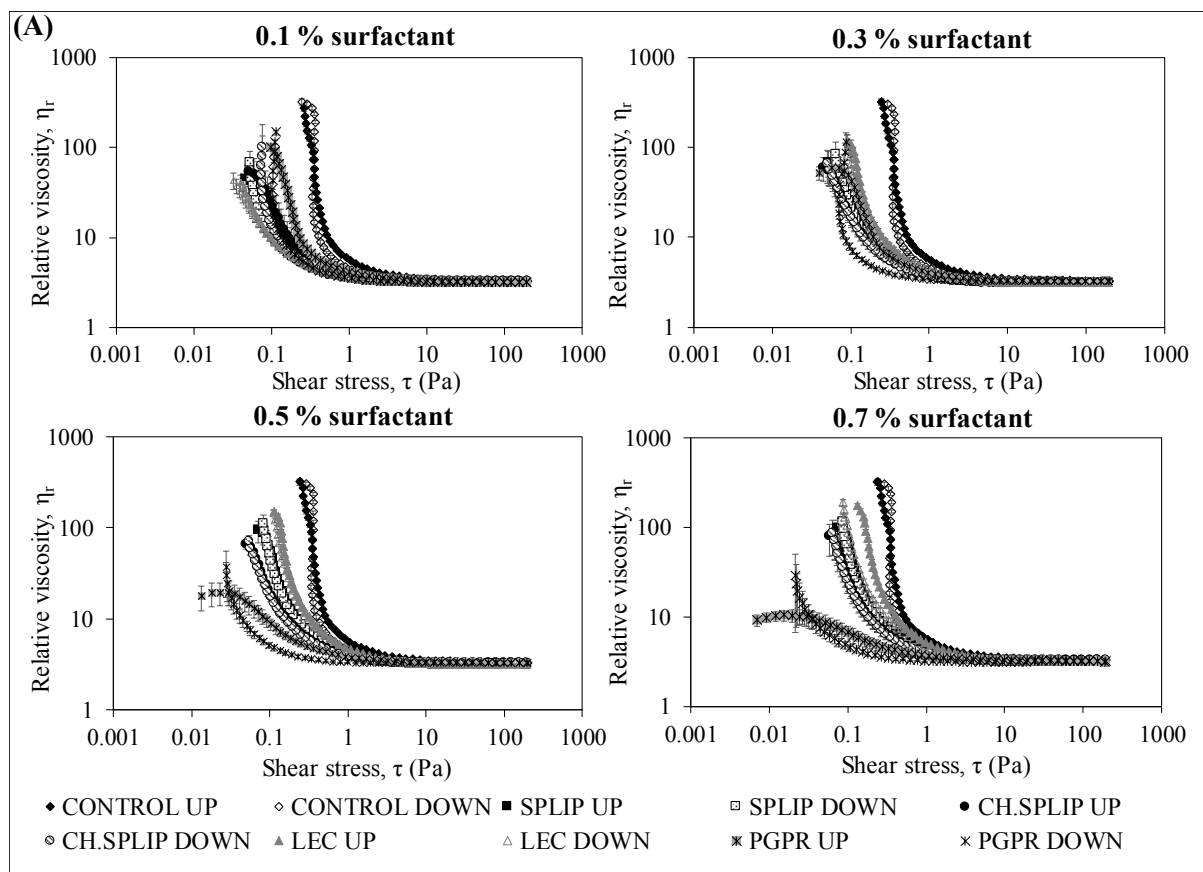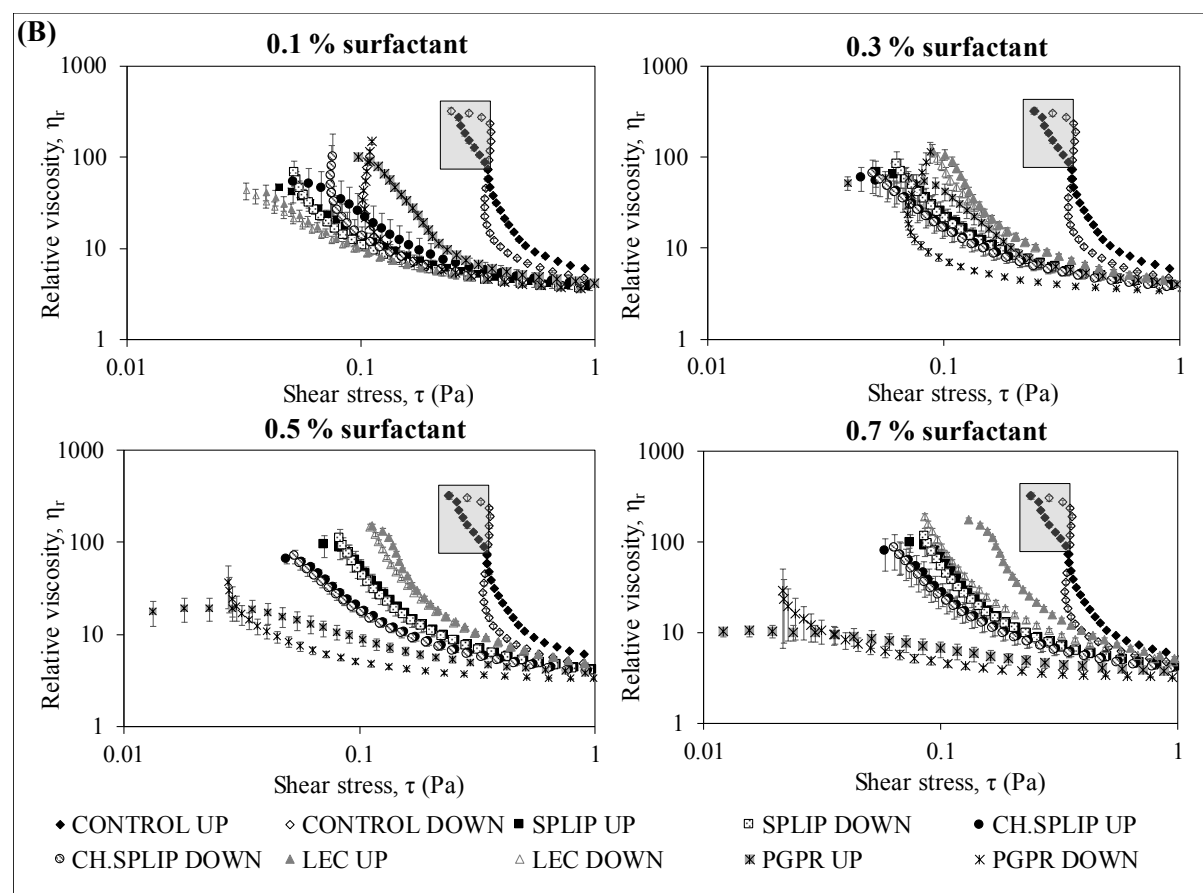
468 **Fig. 4.** (A) Relative viscosity curves of sugar/oil suspensions at sugar volume fraction of 0.28

469 as affected by the concentration of surfactant (mean ± 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

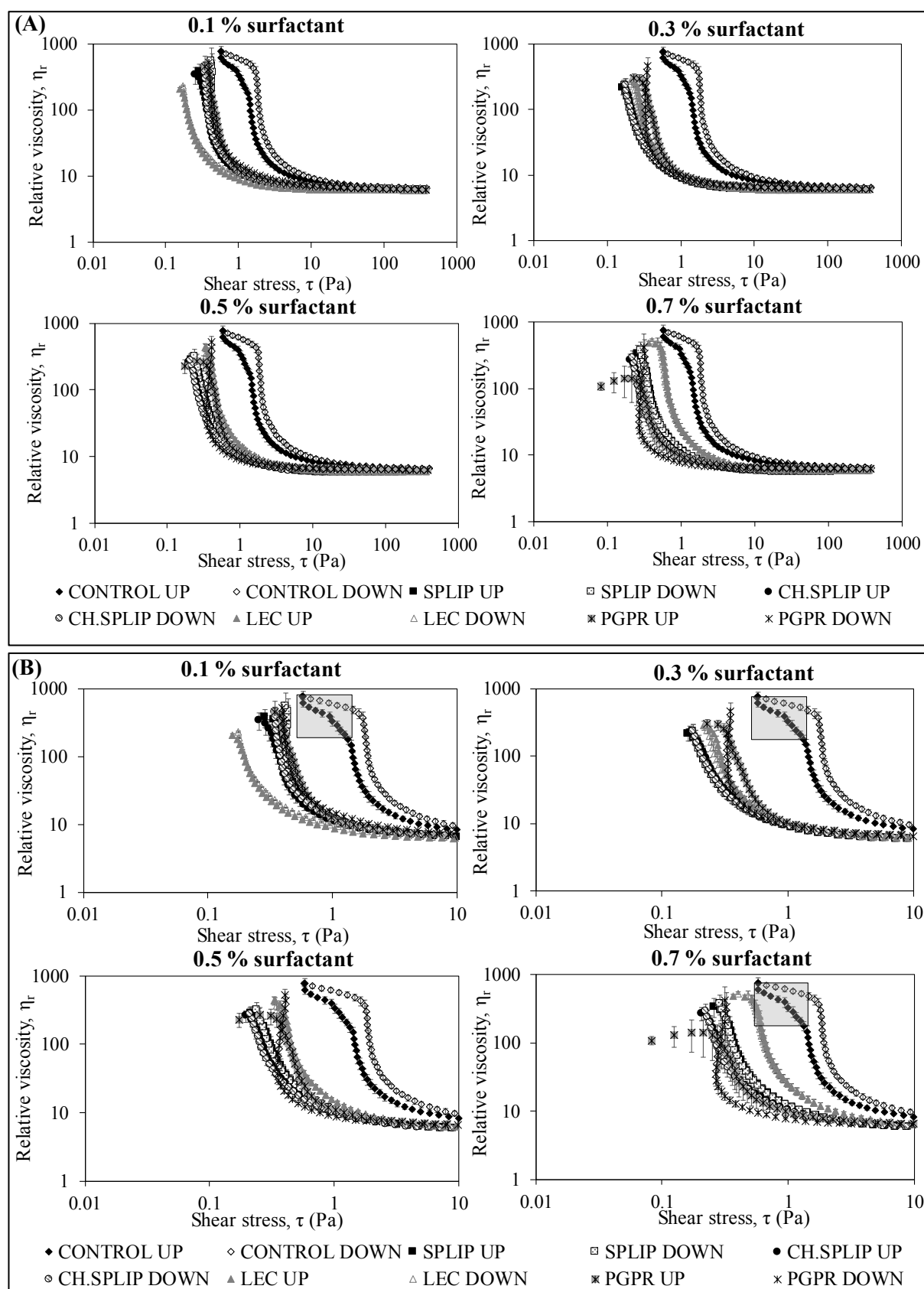472 solid-like to liquid-like behavior.

473

474

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

25

Fig. 6. (A) Relative viscosity curves of sugar/oil suspensions at sugar volume fraction of 0.37

as affected by the concentration of surfactant (mean ± 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} (η40)

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

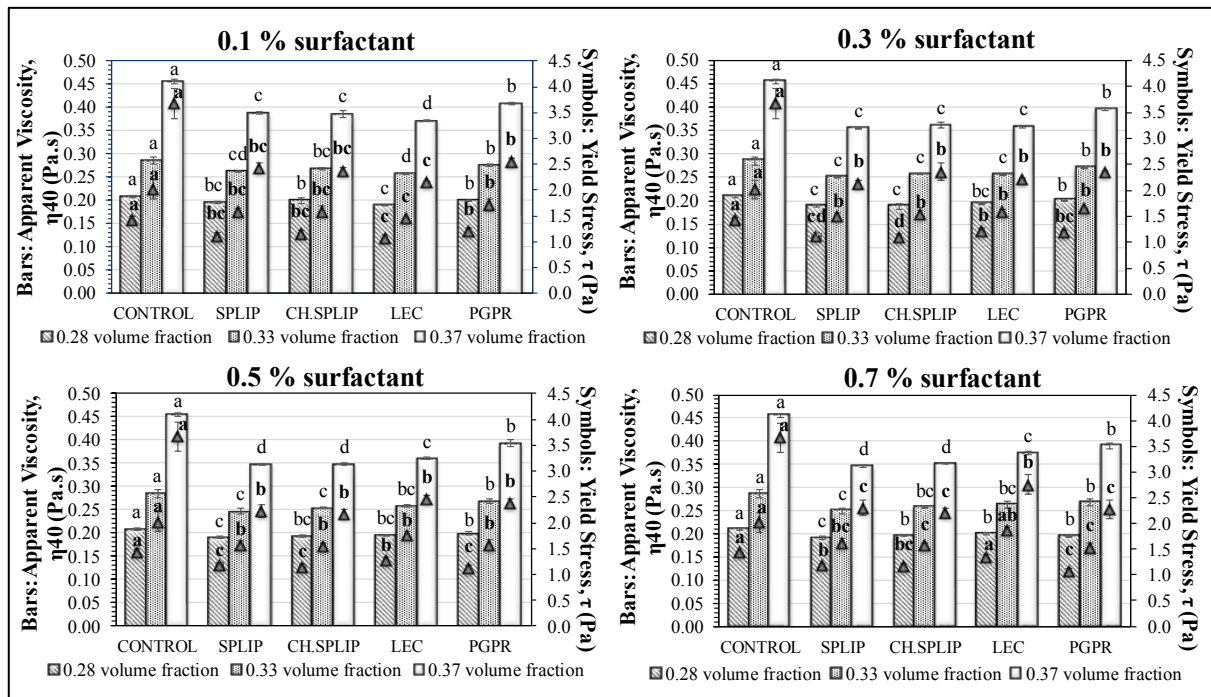568 of the data in view to reformulating chocolate.

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 stearic 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
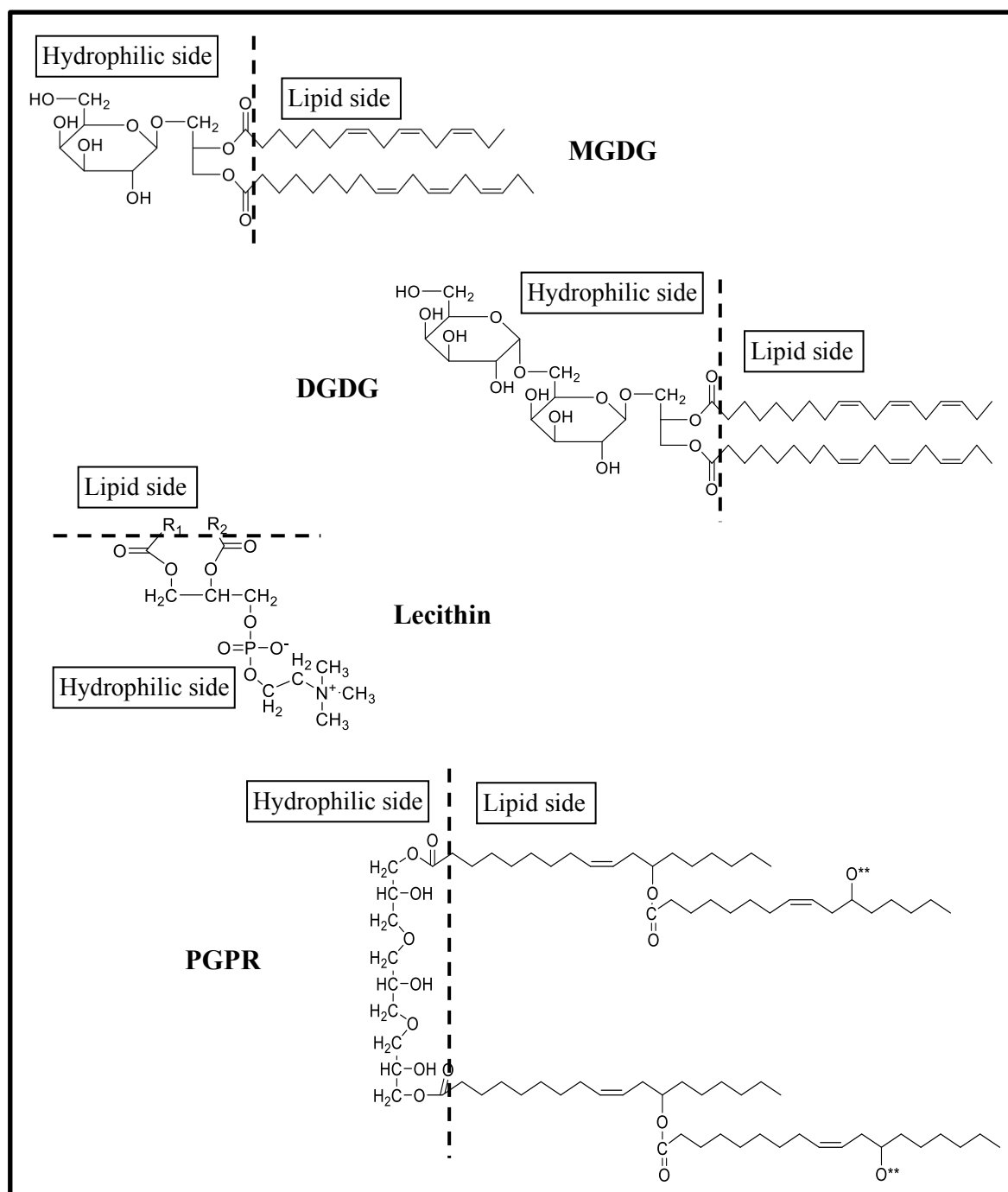
597   pure water (Kobayashi, 2016).

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Fig. 8. Chemical structure of MGDG, DGDG, lecithin and PGPR. R_1 and R_2 are the fatty acids residues. The ** on the PGPR structure denotes polyricinoleic acid chains.

The anionic SQDG and PG have been demonstrated to play an important role in maintaining the bilayer structure of neutral lipids in studies where screening out these lipids 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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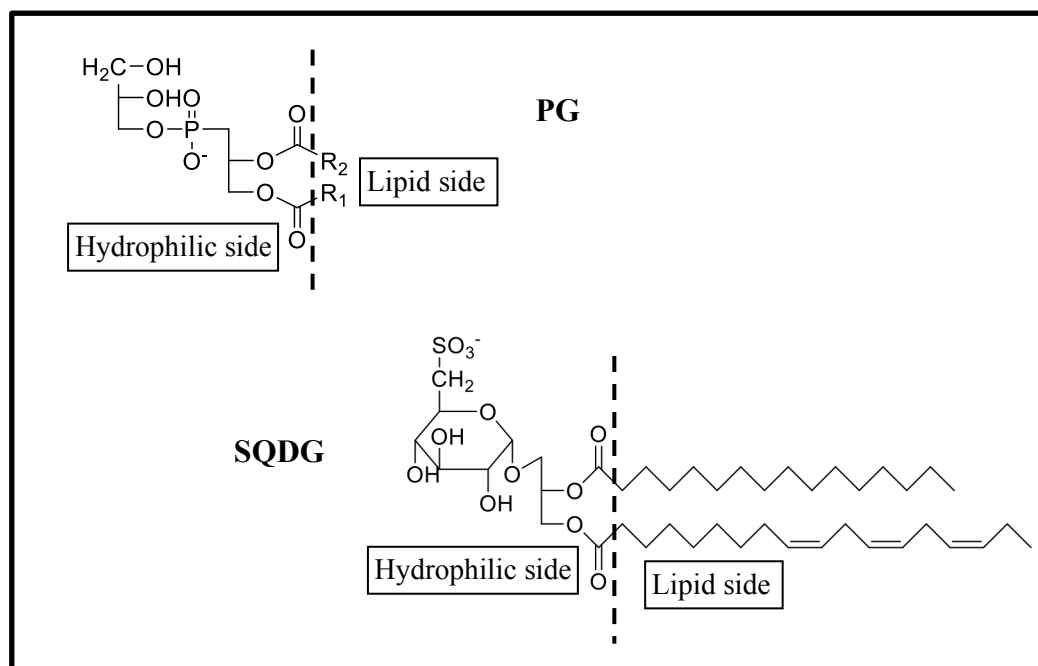
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Fig. 9. Chemical structure of PG and SQDG with the negative charge on the hydrophilic side. R_1 and R_2 denote hydrocarbon chains of fatty acids.

4. Conclusions

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

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

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-

726 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

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

746   df 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-

749   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

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

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

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-

797   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

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

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.Â., & 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. Â.

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. Â. (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-

841   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

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.pd

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.

873

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876