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

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Abstract

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

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

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Lipid extraction from spinach leaf

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

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

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

2.3. Lipid extraction from spinach chloroplast

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

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

2.4. Lipid analysis by Thin Layer Chromatography (TLC)

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

For TLC, the major class of lipids (polar lipids and neutral lipids) were separated using 1-dimensional (1-D) glass HPTLC (high performance TLC) (Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany)), while the polar lipids were separated using 2-dimensional (2-D) glass HPTLC, as follows. A known amount of phosphatidylcholine containing heptadecanoic acid (C17:0PC) as the internal standard was added into the lipid extract to aid analysis with the 1-D TLC plate. The mixture of the lipid sample and the internal standard was spotted (200 µl) onto the 1-D TLC plate and then separated in one direction using 70:30:2 (v/v/v) of isohexane/diethyl ether/formic acid solvent mix. The plate was sprayed with primuline and viewed under UV light. The lipid components were then extracted from the silica plate prior to analysis by GC.

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

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

The band of TLC adsorbent containing lipids was scraped and put into a glass test tube. About 1 ml of toluene and 2 ml of methanolic sulfuric acid (1 %; v/v) were added into the glass test tube. The mixture was then heated to, and held at, 50 °C for 14 – 16 h. After cooling, it was shaken with 2 ml of isohexane and 5 ml of NaCl solution (5 %; w/v). The solvent was then transferred into a new glass test tube. The previous test tube was shaken with another 2 ml of hexane and the two solvents were combined in the new glass test tube. The combined solvents were shaken with 3 ml of $KHCO_3$ solution (2%; w/v). The solvent mixture was then transferred into a new tube and 1 ml of toluene was added before blowing off the solvents into dryness with N_2 gas. After that, isohexane and BHT (butylated hydroxytoluene, antioxidant) were added to give a lipid concentration of 5 mg/ml. The fatty acids methyl esters (FAMEs) were then ready to be injected into the gas chromatograph for further analysis.

2.5. Lipid analysis by Gas chromatography (GC)

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

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

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

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

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

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

2.7. Dynamic interfacial tension

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

2.8. Particle size distribution of icing sugar

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

Table 1

Characteristic size distribution values for the three particle populations of the icing sugar sample used in the sugar/oil suspension systems. The percentage volume differential shows 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

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

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

2.11. Suspension rheology

The rheological properties of the sugar/oil suspensions were evaluated by acquiring 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

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

3. Results and discussion

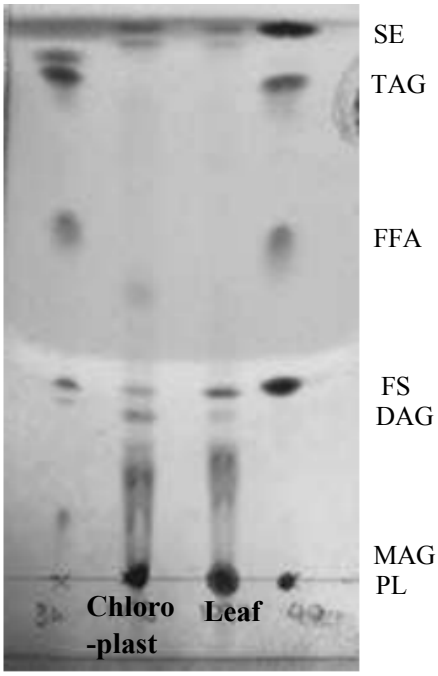
3.1. Yield of lipid extracts

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

3.2. Lipid classes

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

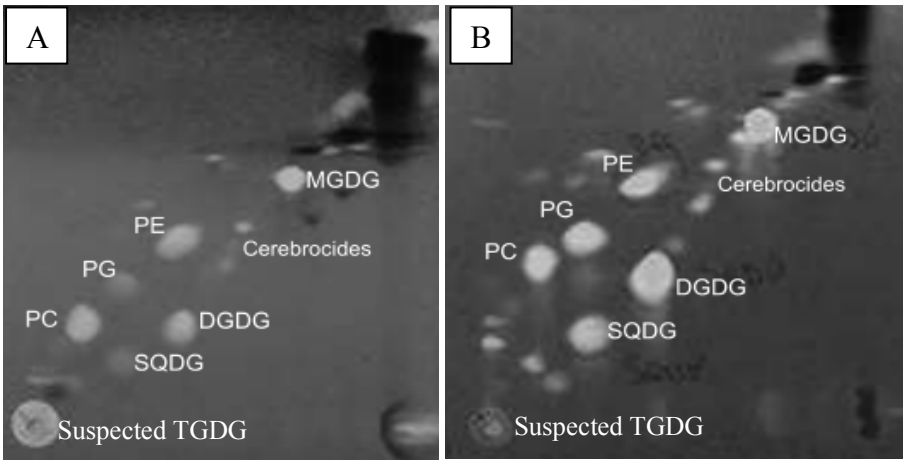


Fig. 2. Polar lipids separated on 2D TLC plate. (A): from spinach leaf; (B) from spinach chloroplast. Abbreviation denote: MGDG = monogalactosyl diacylglycerol, DGDG = digalactosyl diacylglycerol, SQDG = sulfoquinovosyl diacylglycerol, TGDG = trigalactosyl diacylglycerol, PC = phosphatidyl choline, PE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

3.4. Interfacial tension at oil/water interface

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

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

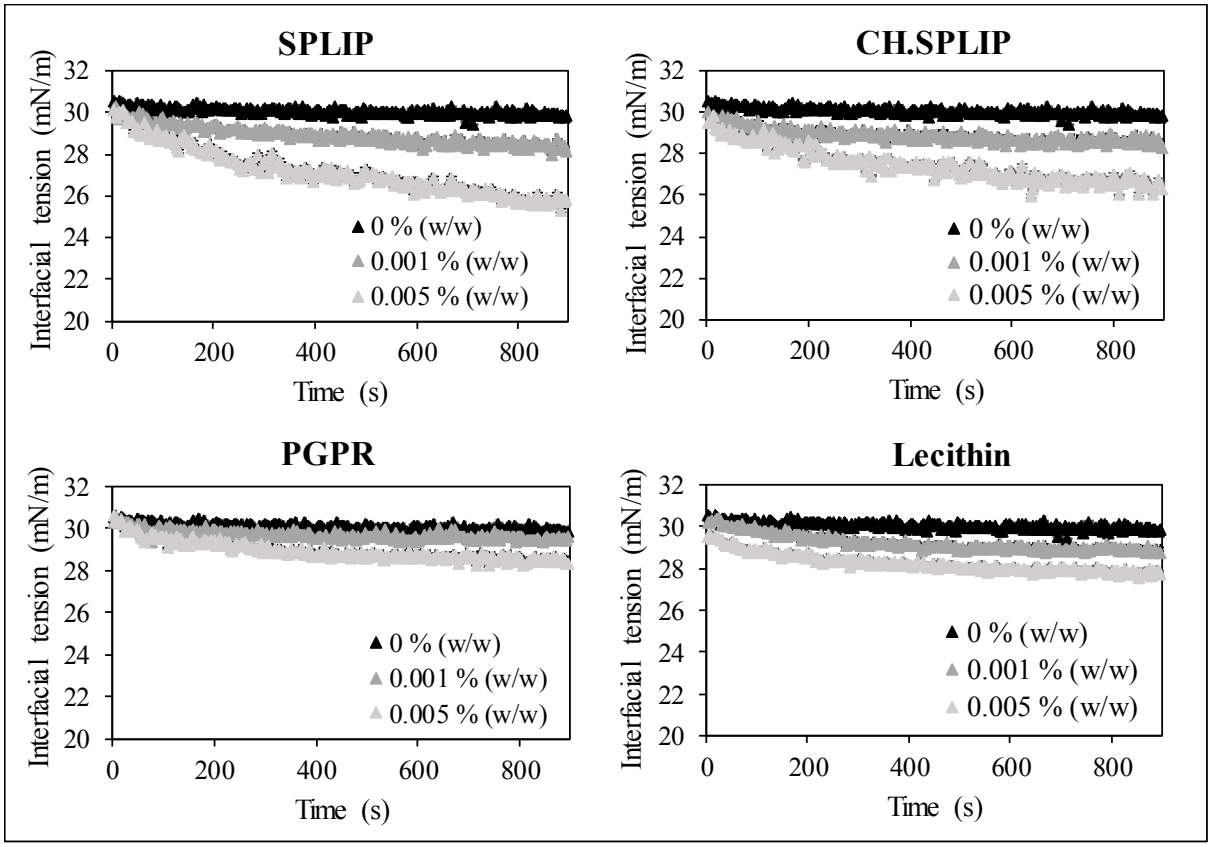


Fig. 3. Surface tension at the water-oil interface of added surfactant either with leaf lipid, chloroplast lipid, lecithin or PGPR. Data plotted as means ± 1 for standard deviation for n=3.

Table 4

Interfacial tension at the oil/water interface (20°C) in presence of spinach lipid extract from leaf or chloroplast, PGPR or lecithin (mean value recorded at 900 s). Different subscript 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

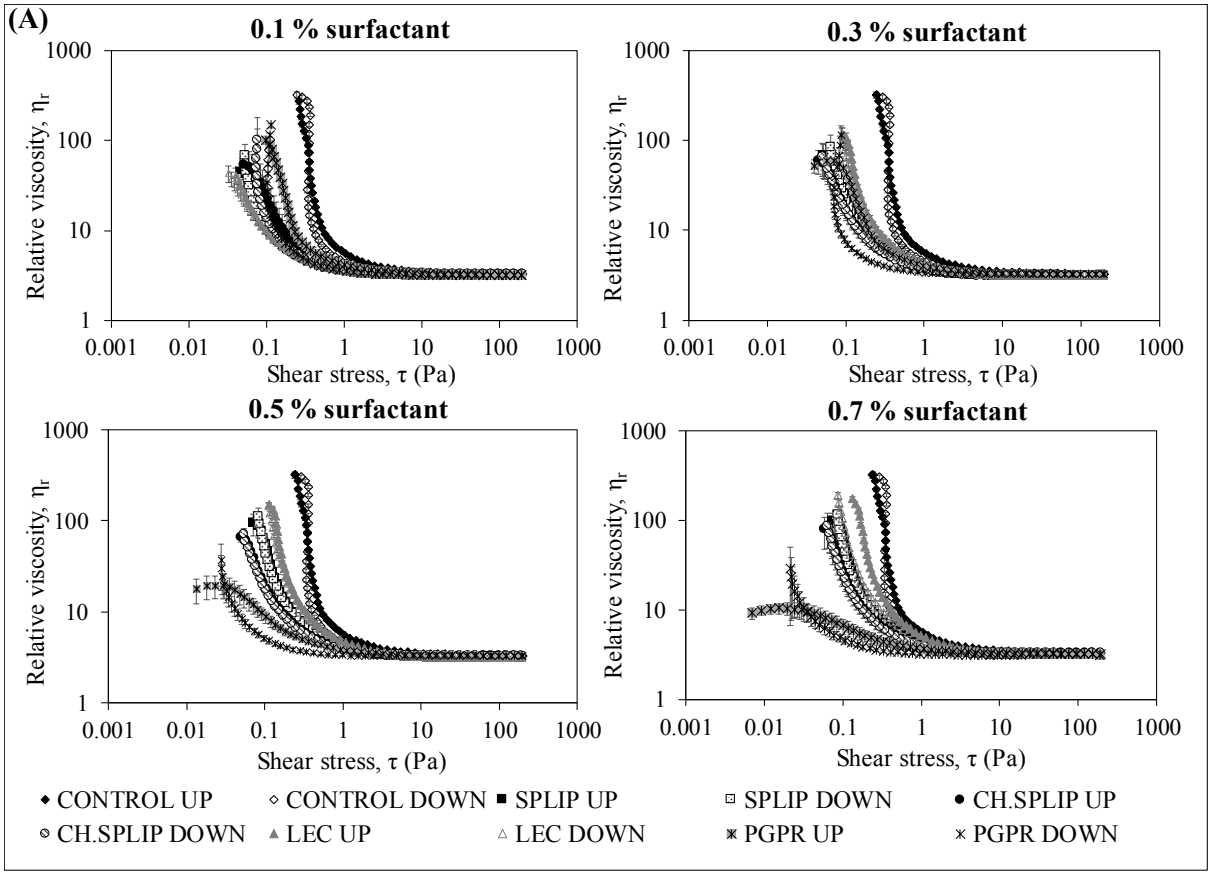
426 3.5. The effect of spinach lipids, PGPR and lecithin on the viscosity profile of sugar/oil
427 suspensions

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.

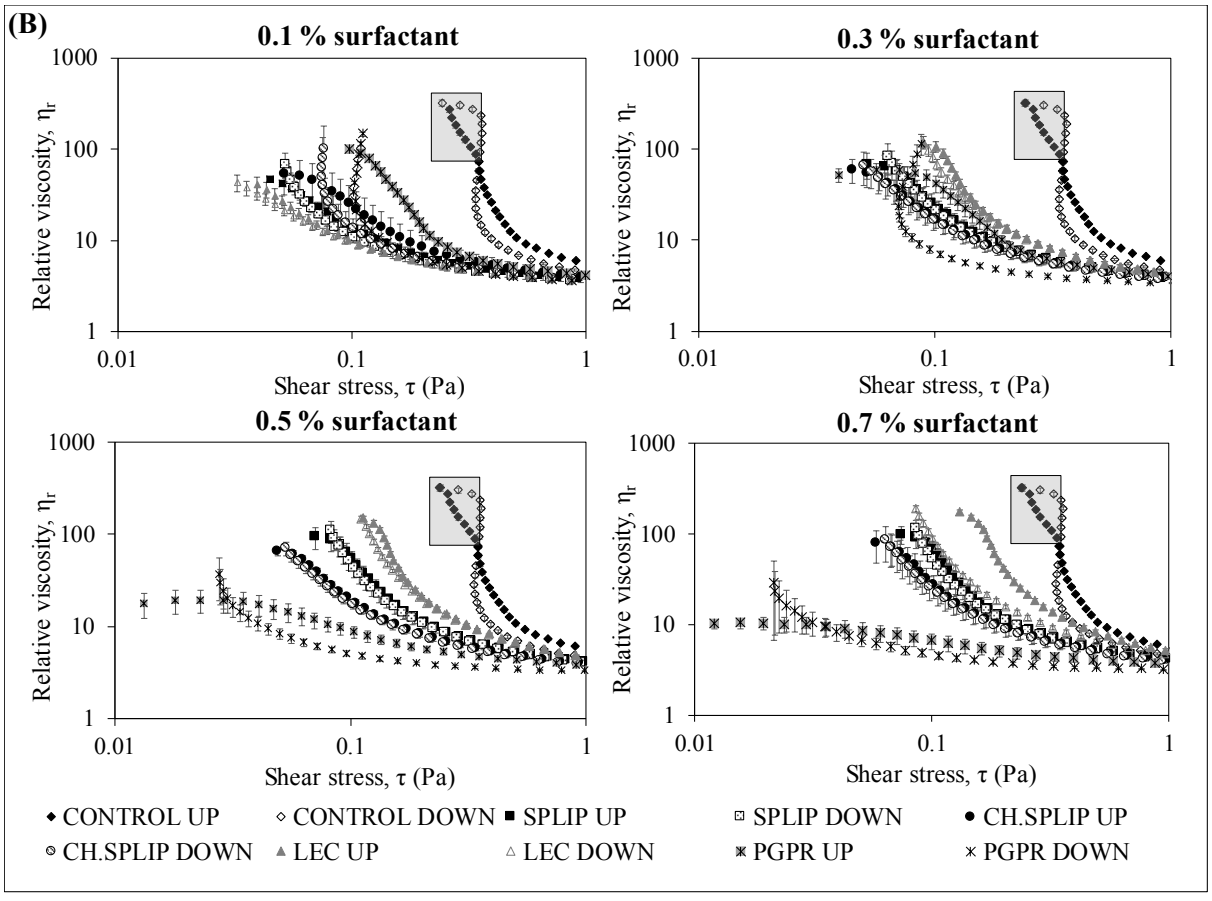
439 As a first observation, irrespective of surfactant system and concentration, the
440 sugar/oil suspensions showed a shear thinning behavior and a transition to a Newtonian
441 plateau at high shear stress. Referring to the increasing shear ramp data, and in accordance
442 with literature (Taylor, 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.

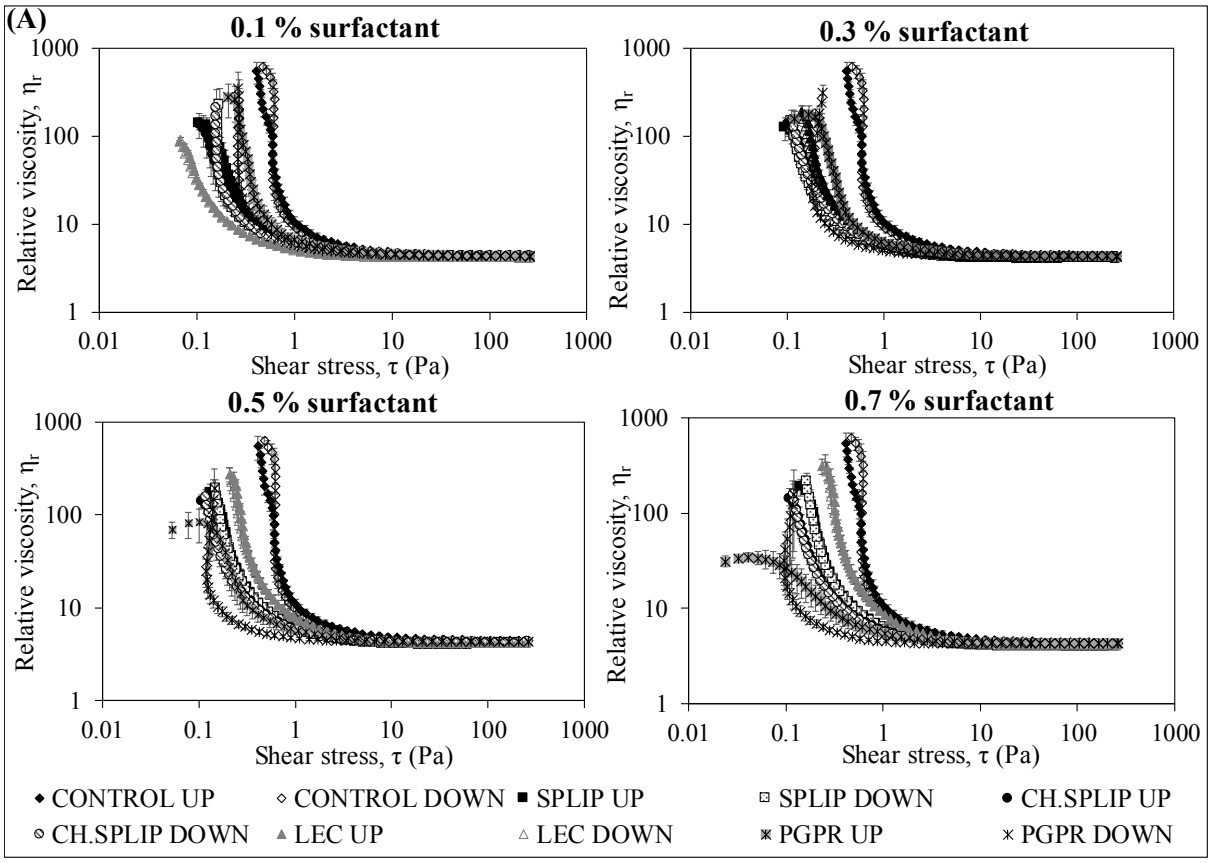

(A)
0.1 % surfactant
0.3 % surfactant
0.5 % surfactant
0.7 % surfactant
Relative viscosity, ηr
Shear stress, τ (Pa)
1000
100
10
1
0.001
0.01
0.1
1
10
100
1000
CONTROL UP
CONTROL DOWN
SPLIP UP
SPLIP DOWN
CH.SPLIP UP
CH.SPLIP DOWN
LEC UP
LEC DOWN
PGPR UP
PGPR DOWN

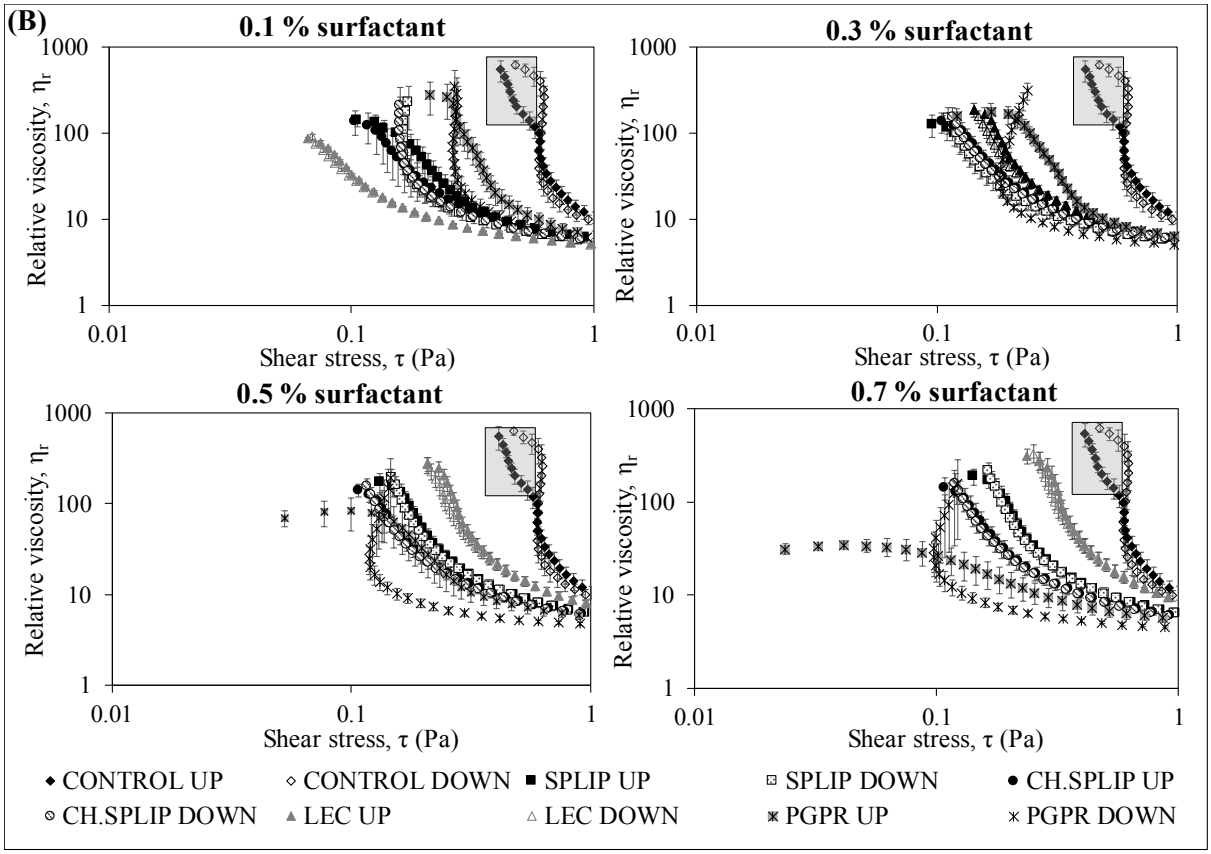
466


(B)
0.1 % surfactant
0.3 % surfactant
0.5 % surfactant
0.7 % surfactant
Relative viscosity, ηr
Shear stress, τ (Pa)
1000
100
10
1
0.01
0.1
1
CONTROL UP
CONTROL DOWN
SPLIP UP
SPLIP DOWN
CH.SPLIP UP
CH.SPLIP DOWN
LEC UP
LEC DOWN
PGPR UP
PGPR DOWN

467

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





475

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 ± 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

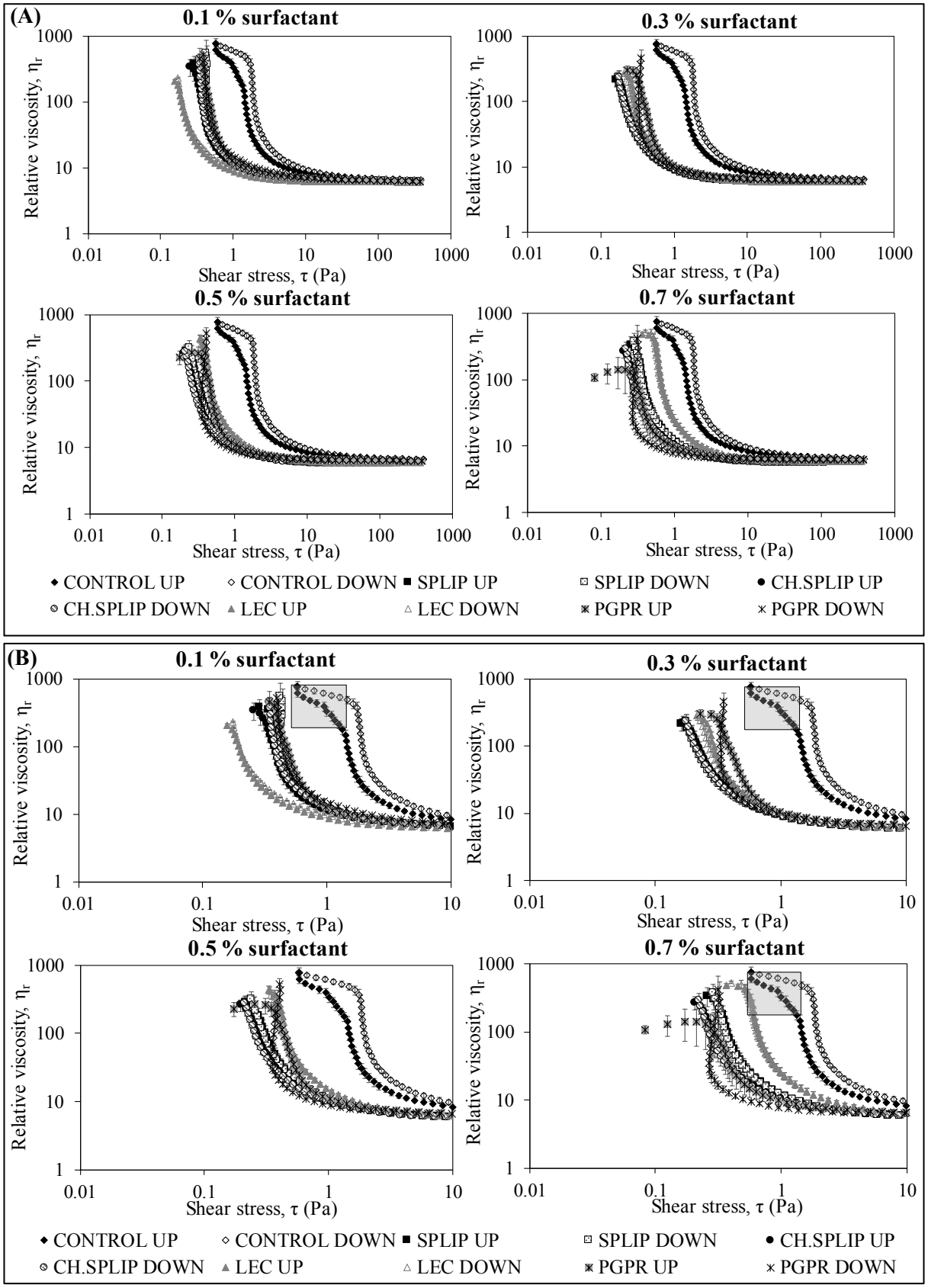


Fig. 6. (A) Relative viscosity curves of sugar/oil suspensions at sugar volume fraction of 0.37 as affected by the concentration of surfactant (mean ± 1.0, n=4). (B) Low shear region data to

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.

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

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

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

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

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

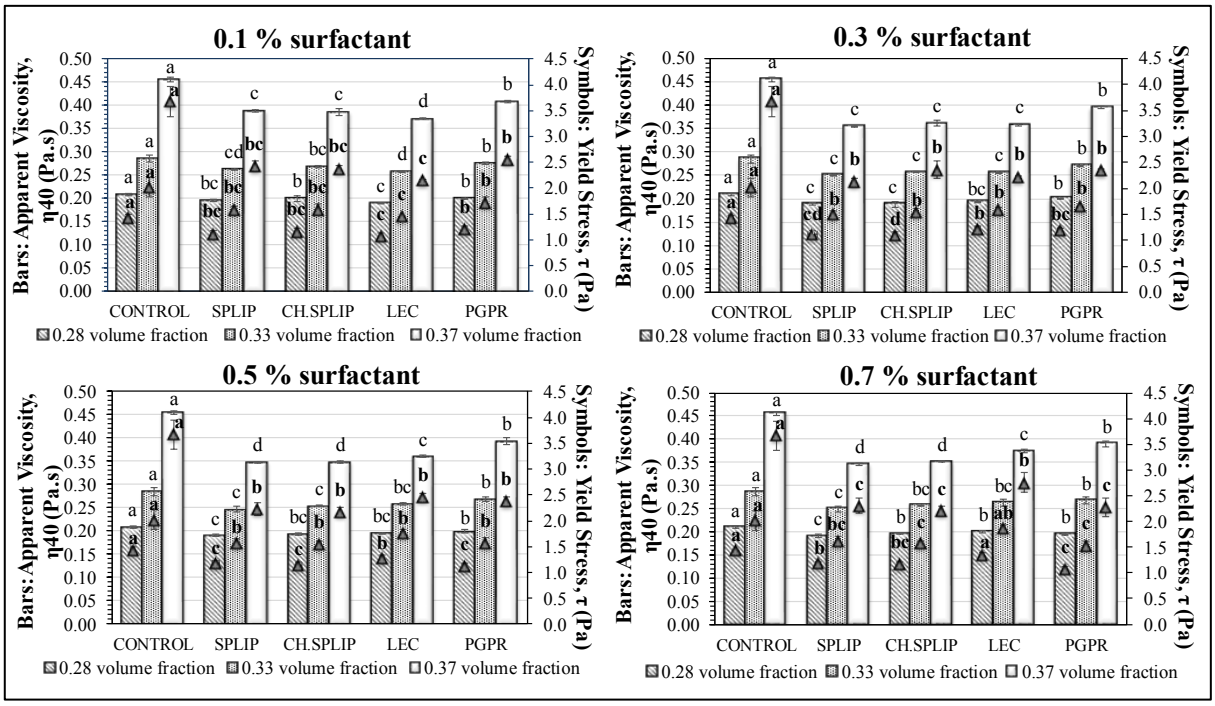
3.6. IOC parameters

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

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

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

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

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

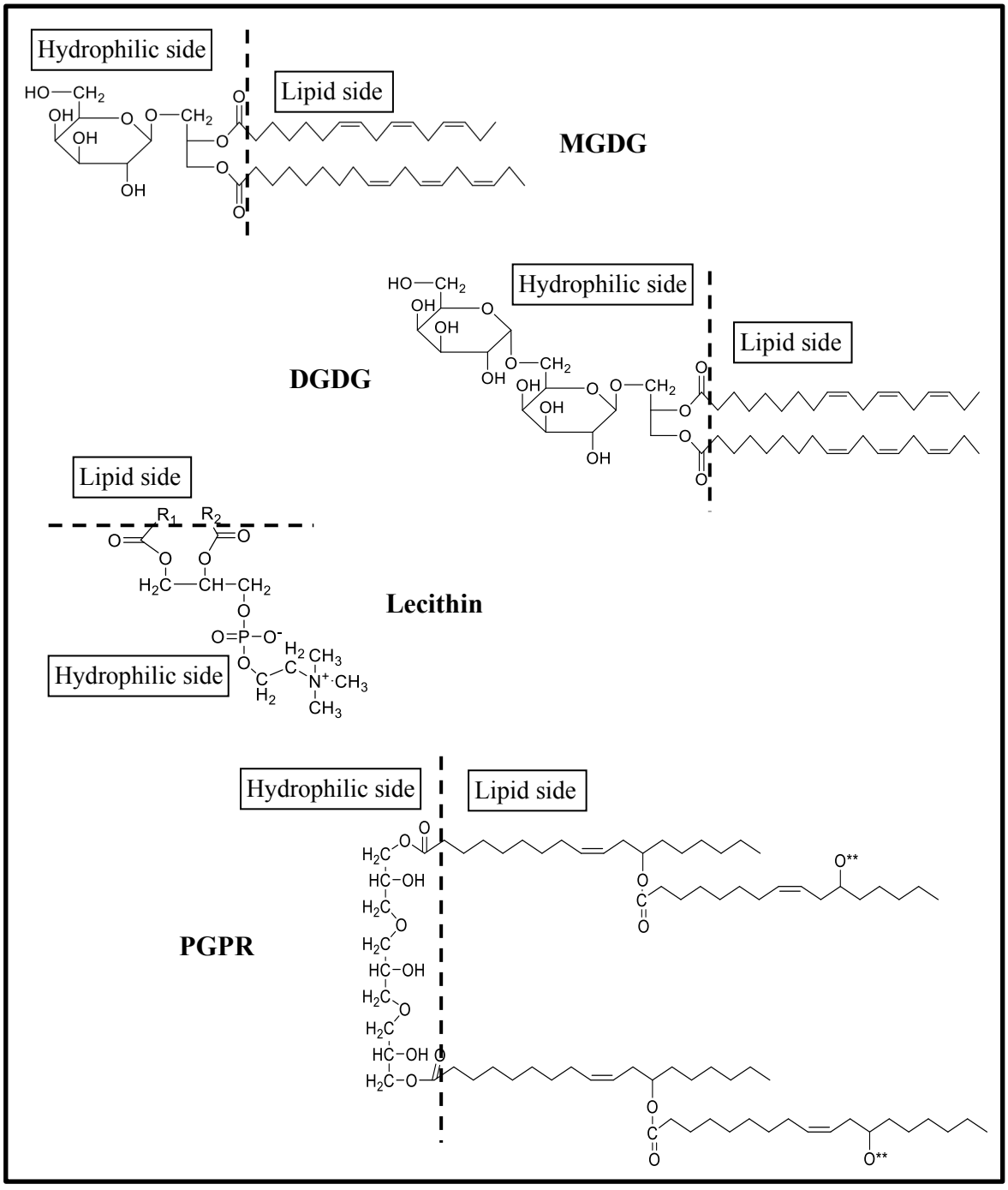


Fig. 8. Chemical structure of MGDG, DGDG, lecithin and PGPR. R_1 and R_2 are the fatty acids residues. The ** on the PGPR structure denotes polyricinoleic acid chains.

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

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

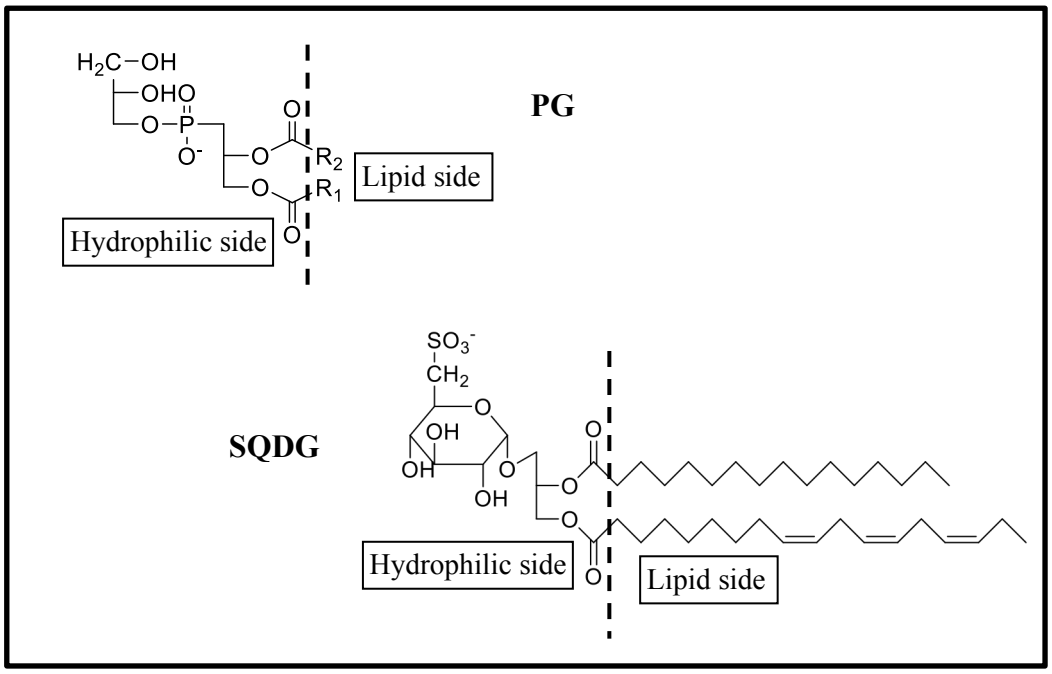


Fig. 9. Chemical structure of PG and SQDG with the negative charge on the hydrophilic side. R_1 and R_2 denote hydrocarbon chains of fatty acids.

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

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

Acknowledgements

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