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Oat and lipolysis: Food matrix effect

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1 ABSTRACT

2 Oat is rich in a wide range of phytochemicals with various physico-chemical,
3 colloidal and interfacial properties. These characteristics are likely to influence human lipid
4 metabolism and the subsequent effect on health following oat consumption. The aim of this
5 work was to investigate the impact of oat materials varying in complexity on the lipolysis
6 process. The composition, structure and digestibility of different lipid systems (emulsions, oil
7 bodies and oil enriched in phytosterols) were determined. The surface activities of
8 phytosterols were examined using the pendant drop technique. Differences in lipid
9 digestibility of the oat oil emulsions and the oil bodies were clearly seen. Also, the digestion
10 of sunflower oil was reduced proportionally to the concentration of phytosterols present. This
11 may be due to their interfacial properties as demonstrated by the pendant drop experiments.
12 This work highlights the importance of considering the overall ~~the~~ structure of the system
13 studied and not only its composition.

14

15 *Keywords:* Oat lipid, food matrix, lipolysis, phytosterols, interface, micelles.

16

17 *Abbreviations:* FFA, free fatty acids; Ocrude, crude oil from oats; OPL4, oat oil with ~4%
18 polar lipids; OPL15, oat oil with ~15% polar lipids; PS, phytosterols; SOs, Sunflower oil
19 treated with Florisil®; WPI, whey protein isolate.

20

21 **Chemical compounds studied in this article**

- 22 β -sitosterol (PubChem CID: 222284); Campesterol (PubChem CID: 173183); Δ 5-avenasterol
23 (PubChem CID: 5281326); Δ 7-avenasterol (PubChem CID: 12795736);
24 Digalactosyldiacylglycerol (PubChem CID: 25203017); Epicoprostanol (PubChem CID:
25 91465); Fucosterol (PubChem CID: 5281328); Monogalactosyldiacylglycerol (PubChem
26 CID: 25245664); Phosphatidylcholine (PubChem CID: 6441487); Stigmasterol (PubChem
27 CID: 5280794).

28 1. Introduction

29 The association between oat and its positive effect on human lipid metabolism, in
30 particular decreases in blood cholesterol levels, has been extensively investigated *in vivo*
31 (Thies, Masson, Boffetta, & Kris-Etherton, 2014). Several mechanisms of action have been
32 proposed linked to the β -glucan contained in oat (Wolever et al., 2010), which includes an
33 increase in viscosity of intestinal contents and interaction with bile salts leading to restricted
34 bile salts recycling (Gunness & Gidley, 2010). However, it is likely that the observed benefits
35 on health are also due to other structural features unique to the oat grain that would dictate
36 the way it behaves during digestion. For instance, our recent *in vitro* data suggests that oat
37 compounds, other than β -glucan, may have an impact on lipid digestibility (Grundy et al.,
38 2017; Grundy, McClements, Ballance, & Wilde, 2018). Oat is rich in macronutrients and
39 bioactives phytochemicals, including arabinoxylans, antioxidants (e.g. phenolic acids,
40 avenanthramides, tocotrienols, and saponins) and phytosterols (Shewry et al., 2008; Welch,
41 2011). Among those constituents, another potential contributor to the positive effect of oat
42 consumption on plasma cholesterol levels could be the phytosterols (Bard, Paillard, & Lecerf,
43 2015). Similarly to β -glucan, the exact processes behind this effect remain unclear, although
44 their interaction with the absorption of cholesterol by displacement of cholesterol from the
45 mixed micelles and formation of mixed crystals leading to cholesterol's precipitation and
46 excretion is currently the strongest explanation (De Smet, Mensink, & Plat, 2012).

47

48 Phytosterols are often studied as isolated compounds, however plant-based foods such
49 as oats are mostly in the form of complex matrices whose constituents interact with each
50 other. The food matrix has been demonstrated to be an important parameter to influence the
51 play a significant role in the functionality of phytosterols (Cusack, Fernandez, & Volek, 2013;
52 Gleize, Nowicki, Daval, Koutnikova, & Borel, 2016). The forms that in which they are

53 delivered to the gastrointestinal tract is probably a crucial element to their bioactivity. Indeed,
54 phytosterol bioavailability and efficacy has been shown to rely on many factors such as the
55 type and quantity of lipids present, the type of phytosterols (i.e. free, esters or steryl
56 glycosides ~~sterol, stanol or ester such as steryl glycoside~~), the source, and the food
57 microstructure (Ostlund, 2002; Alvarez-Sala et al., 2016; Ferguson, Stojanovski, MacDonald-
58 Wicks, & Garg, 2016). In plant-based foods, phytosterols are found as a mixture of free and
59 bound (esters) phytosterols, but not all forms have the same physico-chemical properties and
60 therefore health benefits (Moreau, Whitaker, & Hicks, 2002). Furthermore, phytosterols are
61 hydrophobic and poorly soluble in aqueous solutions, so they associate, mainly via
62 hydrophobic interactions, with various lipophilic structures that are present during digestion,
63 such as oil droplets, lipid vesicles, membranes, and micelles, and this is thought to be critical
64 for their functionality (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000; Amiot et al.,
65 2011).

66
67 Some studies have investigated the fate of the phytosterols during *in vitro* lipid
68 digestion (von Bonsdorff-Nikander et al., 2005; Moran-Valero, Martin, Torrelo, Reglero, &
69 Torres, 2012; Zhao, Gershkovich, & Wasan, 2012; Alvarez-Sala et al., 2016; Gleize, Nowicki,
70 Daval, Koutnikova, & Borel, 2016), but mechanistic studies that could provide information
71 about how they influence lipolysis and micelles formation using different oat matrices are
72 missing. To ~~improve our understanding~~ gain insight into the role played by the broader oat
73 matrix composition and structure on lipid digestion, in this work, ~~we examined~~ various
74 aspects of lipolysis ~~were examined~~, focusing on the contribution made by phytosterols. ~~We~~
75 ~~believe that~~ The kinetics of lipolysis and the mixed micelle formation have important
76 consequences on lipid and cholesterol uptake. During digestion, complex, dynamic self-
77 assembly of amphiphilic and lipophilic molecules occurs, which governs the nature and fate

78 (absorption) of the lipophilic molecules (Phan, Salentinig, Prestidge, & Boyd, 2014). ~~Our~~ The
79 hypothesis of this study was that the oat matrix structure would affect the bioaccessibility and
80 behaviour in solution of the phytosterols and thereby impact lipid digestibility and the
81 formation of mixed micelles. To test this hypothesis, ~~we monitored~~ the lipolysis kinetics of a
82 range of materials with different degrees of complexity was monitored using the pH-stat
83 method. The mixed micelles generated were analysed for particle size and charge. Finally, the
84 effect of phytosterols on the interfacial tension of sunflower oil was also examined using the
85 pendant drop technique.

86

87 2. Materials and Methods

88 2.1. Materials

89 Oat groats (*Avena sativa* L.; variety Belinda) were obtained from Lantmännen
90 Cerealia, Moss, Norway. Oat oils of different purities (OPL4 and OPL15, containing
91 approximately 4 and 15% of polar lipids, respectively; and crude oat oil, Ocrude) were a
92 generous gift from Swedish Oat Fiber (Swedish Oat Fiber AB, Bua, Sweden). Sunflower oil,
93 β -sitosterol (70% purity), epicoprostanol (5 β -cholestan-3 α -ol, 95% purity; used as internal
94 standard), β -sitosterol (95% purity), stigmasterol (95% purity), fucosterol (93% purity),
95 pancreatin (40 U/mg of solid based on lipase activity), bovine bile extract, sodium
96 taurocholate (NaTC, 97%), sodium glycodeoxycholate (NaGDC, 97%), sodium dihydrogen
97 phosphate (99%), disodium hydrogen phosphate (99%), sodium chloride (99.8%), calcium
98 chloride (99%), potassium hydroxide (99.97%), *N,O*-Bis(trimethylsilyl)trifluoroacetamide
99 with trimethylchlorosilane (BSTFA+1% TMCS) were purchased from Sigma (Poole, UK).

100 The internal standards (phosphatidylcholine, PC, phosphatidylethanolamine, PE,
101 phosphatidylinositol, PI, phosphatidylglycerol, PG, lysophosphatidylcholine, lysoPC,
102 digalactosyldiacylglycerol and monogalactosyldiacylglycerol) for phospholipids and

103 galactolipids analysis were supplied by Avanti (Alabama, USA). Pyridine, extra dry (99.5%)
104 was obtained from Fisher Scientific (Loughborough, UK). Campesterol (98% purity), Δ 5-
105 avenasterol (98% purity) and Δ 7-avenasterol (98% purity) were obtained from ChemFaces
106 (Wuhan, China). Powdered whey protein isolate (WPI) was donated by Davisco Foods
107 International (Le Sueur, USA).

108

109 2.2. Material preparation

110 2.2.1. Oat oil bodies

111 Oat groats were ground in a coffee grinder (F20342, Krups, Windsor, UK) and soaked
112 overnight in extraction media (1:5, w/v; 10 mM sodium phosphate buffer pH 7.5, 0.6 M
113 sucrose) as previously described (White, Fisk, & Gray, 2006). The soaked oats were
114 homogenised (Laboratory blender 8010ES, Waring Commercial, USA) at full power for 2
115 min and the slurry filtered through 3 layers of cheesecloth to remove large particles and cell
116 fragments. The filtrate was then centrifuged (Beckman J2-21 centrifuge; fixed rotor JA-10) at
117 20 000 g, 4°C for 20 min. The creamy upper layer was recovered, this is referred to as the oil
118 bodies. The sucrose added to the extraction media facilitated the separation of oil bodies from
119 the rest of the oat constituents (e.g. starch and storage proteins) as it allowed them to float on
120 top of the solution following filtration and centrifugation.

121

122 2.2.2. Oils

123 Sunflower oil (SOs) was treated with Florisil® (Sigma, Poole, UK), which is a porous
124 and absorbent form of magnesium silicate, used to remove polar, surface-active compounds
125 (e.g. phospholipids, galactolipids and sterols) from the oil. Sunflower oil enriched in
126 phytosterols was obtained by mixing the Florisil®-treated sunflower oil with the β -sitosterol
127 from Sigma (70% purity; final phytosterol concentration of 0, 0.5, 1.0, 1.5 and 2.0%) based

128 on a method by Mel'nikov *et al.* 2004. The mixture was heated at 75°C during 15 min under
129 intensive stirring until complete dissolution of the crystalline phase. The solution was cooled
130 down to 25°C for 100 min using a water bath. The oils enriched in phytosterols were used
131 within 5 days to prevent the formation of sterol crystals (checked by light microscopy, *data*
132 *not shown*).

133

134 2.2.3. Emulsions

135 The emulsions were prepared as described in a previous study (Grundy et al., 2017).
136 Briefly, WPI solution was prepared by dissolving 1 wt% of powdered WPI into 10 mM
137 phosphate buffer (pH 7.0 at 37°C) and stirring for at least 2 h. Emulsions were made from
138 either oat oils (Ocrude, OPL15, and OPL4), or Florisil®-treated sunflower oil with or without
139 phytosterols. The emulsions were obtained by pre-emulsifying 1.6 wt% of oil in WPI solution
140 using a homogeniser (Ultra-Turrax T25, IKA® Werke, from Fisher Scientific Ltd.) for 1 min
141 at 1 100 rpm. The pre-emulsion was then sonicated with an ultrasonic processor (Sonics &
142 Materials Inc, Newtown, Connecticut, USA) at 70% amplitude for 2 min.

143

144 2.3. Characterisation of the material

145 Moisture content was determined by weighing 200 mg of oat bodies or oil into
146 microtubes that were placed in a vacuum oven (Townson & Mercer Ltd, Stretford, Greater
147 Manchester, UK) at 40°C for 48 h. The dried samples were then weighed a second time and
148 the moisture content calculated by difference.

149 Total lipid content of the materials was obtained by Folch extraction, fatty acid methyl
150 esters (FAME) derivatisation and Gas Chromatography-Mass Spectrometry (GC-MS; Agilent
151 7890B/5977A GC/MSD, Agilent Technologies, Santa Clara, California, USA) analysis
152 (Grundy et al., 2017).

153 Phytosterol content was determined by a method adapted from a previous study
154 (Alvarez-Sala et al., 2016). Briefly, hot saponification was performed on 100 mg of samples
155 with 1 mL 2 N KOH in ethanol/water (9:1, v/v; 65°C during 1 h), followed by extraction of
156 the unsaponifiable fraction with diethyl ether and derivatisation with BSTFA + 1%
157 TMCS/pyridine (10:3, v/v). The BSTFA derivatives were dissolved in 100 µL of *n*-hexane
158 and analysed by GC. One µL of sample was injected in the GC equipped with a CP-Sil 8 low
159 bleed/MS (50 m, 0.25 mm, 0.25 µm) capillary column (Agilent Technologies, Santa Clara,
160 USA). The oven was initially programmed at 150°C, maintained during 3 min, heated to
161 280°C at a rate of 30°C/min, and kept during 28 min, then raised to 295°C at a rate of
162 10°C/min. Finally, this temperature was maintained for 10 min. The carrier gas was helium
163 (15 psi). The temperature of both the injector port and the **flame ionisation detector** were
164 325°C, and a pulsed split ratio of 1:10 was applied.

165 Quantitative analyses of the polar lipids, i.e., phospholipids and galactolipids
166 (~~phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol,~~
167 ~~lysophosphatidyleoline, digalactosyldiglycerol or monogalactosyldiglycerol~~), were carried out
168 using electrospray ionization tandem triple quadrupole mass spectrometry (API 4000; Applied
169 Biosystems; ESI-MS/MS). The lipid extractions were infused at 15 µL/min with an
170 autosampler (HTS-xt PAL, CTC-PAL Analytics AG, Switzerland). Data acquisition and acyl
171 group identification of the polar lipids were as described in Ruiz-Lopez *et al.* 2014 with
172 modifications. The internal standards for polar lipids were incorporated as: 0.857 nmol 13:0-
173 LysoPC, 0.086 nmol di24:1-PC, 0.080 nmol di14:0-PE, 0.800 nmol di18:0-PI and 0.080
174 di14:0-PG. The standards and 10 µL of sample were combined to make a final volume of 1
175 mL.

176

177 *2.4. Particle size analysis*

178 The droplet size distributions of the oil bodies and the emulsions were measured with
179 a Beckman Coulter LS13320[®] (Beckman Coulter Ltd., High Wycombe, UK). Water was used
180 as a dispersant (refractive index of 1.330), and the absorbance value of the oil droplets was
181 0.001. Crude oat oil had a refractive index of 1.463, OPL15 1.470, and OPL4 and sunflower
182 oil 1.473 as measured using a refractometer (Rhino Brix90 Handheld Refractometer,
183 Reichert, Inc., New York, USA). The particle size measurements were reported as the
184 surface-weighted mean diameter ($d_{3,2}$).

185 The micelles formed after 1 h digestion, with (digested) and without (blank) enzyme,
186 were obtained by centrifuging the digesta at 2 200 g for 1 h at 10°C and filtrating the aqueous
187 fraction through 0.8 µm and then 0.22 µm filters (Gleize, Nowicki, Daval, Koutnikova, &
188 Borel, 2016). The average size and zeta-potential of the micelles were determined with a
189 Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK).

190 Values of particle size (volume or intensity percentage) are presented as the means ±
191 SD of at least three replicates.

192

193 2.5. *In vitro* duodenal digestion (pH-stat)

194 The rate and extent of lipolysis of oil bodies, oat oils (Ocrude, OPL15, and OPL4) and
195 sunflower oil containing various amounts of phytosterols were continuously measured by
196 titration of released free fatty acids (FFA) with 0.1 M NaOH at 37°C and an endpoint of pH
197 7.0. The details of the *in vitro* duodenal digestion model used can be found elsewhere
198 (Grundy et al., 2017). The final composition of the reaction system was 0.8 wt% lipid (300
199 mg of lipid from oil bodies or emulsion prepared as in Section 2.2.3.), 12.5 mM bile salts, 2.4
200 mg/mL lipase, 150 mM NaCl and 10 mM CaCl₂. All lipolysis experiments were carried out in
201 triplicate.

202

203 2.6. *Interfacial measurements*

204 The interfacial tension at the oil/water interface was measured using the pendant drop
205 technique with a FTA200 pulsating drop tensiometer (FirstTen Angstroms, Portsmouth, VA)
206 as previously described (Chu et al., 2009). An inverted oil drop was formed at the tip of a
207 Teflon-coated J-shaped needle (internal diameter of 0.94 mm) fitted to a syringe with a total
208 volume of 100 μ L. The oil drop was formed in a glass cuvette containing 5 mL of 2 mM bis-
209 tris buffer, 0.15 M NaCl, and 0.01 M CaCl₂, at pH 7 and maintained at 37°C. The
210 measurements were repeated in presence of bile salts (9.7 mM of mixed NaTC and NaGDC,
211 53 and 47% respectively) and during lipolysis, in conditions that simulated the physiological
212 environment of the duodenum (9.7 mM bile salts, 15 μ M lipase and 75 μ M colipase). The
213 initial droplet formed had a volume between 50 and 3 μ L depending on the experimental
214 conditions (i.e. amount of phytosterols, presence of bile salts and lipase). The images were
215 captured for 1 h or until the oil drop detached from the tip of the J-shaped needle due to the
216 large decrease in interfacial tension. The shape of the drop in each image was analysed by
217 fitting the experimental drop profile to the Young-Laplace capillarity equation. Each set of
218 experiments was performed in triplicate.

219

220 2.7. *Microstructural analysis*

221 The microstructure of the oat groats and oil bodies was studied using either optical
222 (Olympus BX60, Olympus, Southend-On-Sea, UK) or scanning electron (SEM; Zeiss Supra
223 55 VP FEG, Cambridge, UK) microscopes. For optical microscopy, samples of oil bodies at
224 baseline, and before and after digestion, were stained with Nile red (1 mg/mL in dimethyl
225 sulfoxide) and then mounted on a glass slide, covered and viewed immediately. Oat groats
226 observed by SEM were prepared as presented elsewhere (Grundy et al., 2017).

227

228 2.8. Statistical analysis

229 The data were analysed using SPSS version 17.0. For all tests, the significance level
230 was set at $p < 0.05$ (2 tailed). The differences between the lipolysis of sunflower oil alone
231 (SOs), and SOs enriched in phytosterols and the oat materials (i.e. oil bodies and oils) were
232 analysed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test.
233 ζ -Potential of the micelles was analysed by one-way ANOVA followed by Tukey's post-hoc
234 test and Student's paired *t*-test was used to evaluate differences between the blank and
235 digested samples.

236

237 3. Results and Discussion

238 3.1. Characterisation of the oat and sunflower materials

239 Table 1 shows that the three oat oils were made of the same types of lipids
240 (triacylglycerides, phospholipids, galactolipids and phytosterols), but differed in the
241 proportion of these compounds. In particular, Ocrude and OPL15 contained about 14% of
242 phospholipids whereas OPL4 only 3.6%. Galactolipids and phytosterols were found in much
243 lower quantity than phospholipids, between 0.4 and 1.2 g in 100 g of oil for galactolipids and
244 between 224 and 245 mg in 100 g of oil for phytosterols. The oil bodies contained only ~50 g
245 of lipids for 100 g which is in agreement with a previous study (White, Fisk, & Gray, 2006).
246 Compared with the oat oils, oil bodies contained lower amount of phytosterols (~230 and 75
247 mg, for the oils and oil bodies, respectively), suggesting that the phytosterols are present
248 mainly in the oil phase although a small amount may be embedded within the monolayers of
249 phospholipids and proteins (oleosin) (Chen, Cao, Zhao, Kong, & Hua, 2014). A significant
250 proportion of the phytosterols present in the oils could have originated from the cell
251 membranes when extracting the oil from the oat tissue (Hartmann, 1998). The phytosterols
252 would have therefore existed in both esterified and free forms as they would have different

253 physico-chemical properties and thereby they would partition between the membrane and the
254 oil phase (Moreau, Whitaker, & Hicks, 2002).

255 The average droplets size of the emulsions made from the oat oils increased in the
256 following order: OPL4 (2.0 μm) < SOs (2.4 μm) < Ocrude (3.4 μm) < oil bodies (4.5 μm) <
257 OPL15 (4.6 μm) (Figure 1A). The differences observed between Ocrude and OPL15 are
258 unexpected given the similarity in their composition (Table 1). On the other hand, emulsions
259 made from sunflower oils containing increasing amounts of phytosterols had comparable
260 particle size distributions, on average 2.2 μm (Figure 1B). The differences in the lipid
261 composition of the oils do not explain the variability observed in the particle size
262 distributions. Therefore, for identical emulsification methods, the lipid type and quantity are
263 not the only parameters influencing the droplet size of the emulsions. Other constituents of
264 the oils, even though present in minute amount, may have altered the interaction(s) between
265 the components of the emulsion and thereby the emulsification process (e.g. tocopherols and
266 minerals such as calcium). Addition of α -tocopherols to an emulsion made from milk lipids
267 was shown to increase the size of the emulsion droplets (Relkin, Yung, Kalnin, & Ollivon,
268 2008). It is therefore possible that certain bioactives present in oat oil may have crystallised
269 during homogenisation, which could have led to droplet aggregation (McClements, 2012).
270 Moreover, the presence of calcium in the emulsion preparation before homogenisation has
271 been shown to influence the concentration of proteins at the surface of the emulsion droplets,
272 and thereby the droplets size (Ye & Singh, 2001). The droplet size would also have been
273 affected by the viscosity of the oil phase (Cornec et al., 1998), which could have been
274 influenced by the different lipid compositions.

275 Microscopy images revealed that the oil bodies isolated after centrifugation formed
276 some aggregates (Figures 2B1 and B2). The median oil body diameter was higher (4.5 μm)
277 than the ones previously reported (1.1 μm) (White, Fisk, & Gray, 2006). This difference in

278 size may be due to the fact that in the current study oil bodies were not all separated from
279 each other. We hypothesized that some compounds released during the extraction of the oil
280 bodies, perhaps specific to the Belinda oat variety (at least in quantity), may have interacted
281 with the lipid droplets and caused aggregation. Indeed, it has been demonstrated in rapeseed
282 that **the size of the oil bodies** varied between plant varieties as well as **with** the nature and
283 composition of phospholipids and sterols, which may affect the stability of the oil bodies
284 (Boulard et al., 2015). Fusion of the oil bodies has also been observed in oat grains when the
285 amount of oleosins embedded within the monolayer coating the oil bodies was low, in
286 particular for mature grains (Heneen et al., 2008). On the contrary, in the present study the oil
287 bodies did not appear to coalesce in the oat endosperm (Figures 2A1 and A2), but they tended
288 to flocculate once extracted from the oat matrix (Figures 2B1 and B2). This phenomenon was
289 recorded in our recent work where depletion flocculation of sunflower oil droplets was
290 induced by the β -glucan released from the oat matrix following incubation of oat flakes and
291 flour (Grundy, McClements, Ballance, & Wilde, 2018). However, no β -glucan was detected
292 in the oil body preparation when stained with a dye specific to the polysaccharide, i.e.,
293 calcofluor white (*data not shown*). **Similarly, starch granules were completely removed**
294 **during the oil body extraction and therefore not present in the final preparation as revealed by**
295 **staining with iodine. Some of the oil bodies may resemble starch granules in appearance, but**
296 **they were undeniably lipids as showed in Figure S1 of the supplementary material where all**
297 **the particles in the image were stained with Nile red, and therefore lipid.** On the other hand,
298 toluene blue staining indicated the presence of proteins (**Figure S2 of the** supplementary
299 material). Therefore, it is likely that complex interactions formed between some of the oat
300 components when disturbing the oat matrix during extraction, thereby making the isolation of
301 individual oil bodies challenging.

302

303 3.2. Digestibility experiments using the pH-stat method

304 3.2.1. Lipolysis kinetics

305 Despite having different emulsion droplets sizes (Figure 1A), OPL15 and Ocrude had
306 the same lipolysis kinetics ($p = 0.970$, Figure 3A). OPL4 emulsion was digested to the same
307 extent ~~than~~ as the control sunflower oil ($p = 0.806$). The purification process of the oils, and
308 the polar lipid concentration (i.e. phospholipids and galactolipids) could have altered the
309 interactions between their constituents in the baseline material and thereby affected their
310 behaviour during digestion (e.g. prevent change of phase - crystallisation - at the interface).

311 **Oil** bodies extracted from almond have been found to be rapidly digested when in
312 presence of pancreatin, containing proteases and phospholipase, that can hydrolyse the oil
313 bodies membrane allowing the lipase to easily access the triacylglycerols (Beisson et al.,
314 2001; Grundy et al., 2016). However, in the current work, the oil bodies appeared to be the
315 least digestible substrate ($p < 0.005$) with only 7.4 mmol/L of FFA produced compared with ~
316 10.0 and 12.8 mmol/L for Ocrude and SOs, respectively (Figure 3A). The fact that some oil
317 bodies aggregated during their extraction from the oat groats, and ~~that the formation of~~ flocs
318 ~~were formed~~ when they **oil bodies** were mixed with the digestion reagents (WPI solution, bile
319 salts, and electrolytes; Figures 3C1 and C2) are likely to explain these results. Indeed, an
320 increase in droplet size diminishes the surface area **per unit volume** of the lipid phase and
321 thereby affects the ability of the lipids to be hydrolysed (Reis, Watzke, Leser, Holmberg, &
322 Miller, 2010). Almond oil bodies have been shown to form similar structures during gastric
323 digestion ~~than to~~ the ones observed in Figure 3C, some of the almond proteins being resistant
324 to pepsin activity (Gallier & Singh, 2012). Hence, the network formed by a combination of
325 compounds (possibly proteins, phytosterols, galactolipids, saponins, and phospholipids)
326 around, or at the vicinity, of the droplets is likely to have hindered the access of the lipase to
327 its substrate (Chu et al., 2009; Grundy, McClements, Ballance, & Wilde, 2018). Flocculated

328 oil bodies could be clearly seen in Figures 3C3 and C4 confirming that these newly formed
329 structures were difficult to degrade.

330 Many compounds found in oat may be responsible for the resistance to digestion of its
331 oil and oil bodies. Given the recognised positive impact of phytosterols on lipid metabolism
332 (De Smet, Mensink, & Plat, 2012; Bard, Paillard, & Lecerf, 2015), we chose to investigate
333 specifically their effect on lipolysis in a more controlled way by adding increasing quantities
334 (0 to 2%) to sunflower oil. A decrease in lipid digestibility, proportional to the concentration
335 of phytosterols in the oil, was recorded with 10.5 mmol/L of FFA produced for the oil
336 containing 2% of phytosterols (Figure 3B). Published *in vitro* studies examining the impact of
337 phytosterols on lipolysis are scarce. One group found that disodium ascorbyl phytostanol
338 phosphate, but not stigmastanol, was able to reduce the extent of lipid digestion possibly by
339 competing with bile salts for occupying the interface (Zhao, Gershkovich, & Wasan, 2012).
340 In a human study, the addition of phytosterol esters to a meal did not modify lipid digestion in
341 the duodenum (Amiot et al., 2011). The authors also showed their poor solubility in mixed
342 micelles or small vesicles. The fact that the phytosterols were esterified may explain this
343 finding. However, phytosterol esters would be converted into free sterols in the human
344 duodenum via the activity of carboxyl ester hydrolase (Gleize, Nowicki, Daval, Koutnikova,
345 & Borel, 2016).

346 Overall, the reduction in the extent of lipolysis induced by the phytosterols added to
347 the sunflower oil was less important than for some of the oat materials (i.e. oil bodies, Ocrude
348 and OPL15), even though the latter contained much less phytosterols (2 g in the SOs
349 compared with an average of ~0.3 g for the oat oils, Table 1). This implies that the diminution
350 in digestibility of lipids from oat is more likely to be due to a combination of processes, some
351 of which involving phytosterols.

352

353 3.2.2. *Micelles characterisation*

354 In order to shed some light on the possible mechanism(s) behind the reduction in lipid
355 digestibility in presence of some of the materials studied here, the micelles in the aqueous
356 phase were isolated and analysed for size and charge. The micelles are important for
357 transporting the lipolytic products away from the oil phase, and so they play a key role in the
358 lipid digestion process. Clear differences were observed in the ζ -potential and size of the
359 micelles produced from either blank (control experiments without enzyme) or digested
360 samples (Figure 4). The particle size distribution of the micelles showed, for all blank
361 samples, two peaks: one around 5 nm and a second one around 200 nm (Figures 4A1 and
362 B1). Interestingly, the micelles in the blank oil bodies sample had another size peak at 11-12
363 nm. ~~Following Digestion~~ with the addition of enzymes resulted in a dramatic shift towards
364 the formation of the larger micelles of more homogeneous size (~150 nm). For all samples,
365 apart ~~for~~ from the digested SOs and oil bodies, the micelle population at ~5 nm diameter
366 completely disappeared (Figures 4A2 and B2). To investigate the effect of phytosterols, the
367 experiment was repeated in the presence of increasing concentrations of phytosterols (Figures
368 4B1 and B2). All the blank samples again showed the two populations around 5 nm and 200
369 nm. However, following the addition of the enzymes, for all samples containing phytosterols,
370 the population at ~5 nm diameter completely disappeared, suggesting that the micellar
371 behaviour of the different oils during digestion was strongly influenced by the presence of
372 phytosterols.

373 Regarding the ζ -potential, the micelles from the oil bodies samples also had lower
374 values (-9.2 and -18.0 mV for blank and digested samples, respectively) compared with the
375 other materials (overall about -15.6 and -28.6 mV for blank and digested samples,
376 respectively) (Figures 4C1 and C2). The lower charge recorded for the micelles of the oil
377 bodies reflects the disparity in the initial structure, and thereby digestibility, between this

378 complex material and the emulsions which resulted in the formation of mixed micelles of
379 different sizes and compositions. The ζ -potential values of the micelles obtained from the
380 digestion of emulsions containing phytosterols are in disagreement with other studies, i.e.,
381 below -45 mV down to -65 mV (Rossi, Seijen ten Hoorn, Melnikov, & Velikov, 2010; Nik,
382 Corredig, & Wright, 2011). The characteristics of the emulsion (e.g. starting droplet size and
383 emulsifier) and the digestion models used may explain the discrepancy in the micelles
384 generated during lipolysis. An important observation here is the complete disappearance of
385 the 5 nm population following digestion of samples with any significant levels of
386 phytosterols. The smaller, 5 nm population is likely to be comprised mainly of simple bile
387 salt micelles, and the larger population will be mixed micelles and/or vesicles. The bile salts
388 in the small micelles can exchange rapidly with those in solution and adsorbed to the
389 interface, leading to the rapid adsorption and desorption kinetics (Parker, Rigby, Ridout,
390 Gunning, & Wilde, 2014). This is thought to be responsible for the ability of bile salts to
391 remove lipolytic products from the interface. The disappearance of the small micelle peak
392 suggests that the equilibrium between the different populations has changed, shifting towards
393 the larger population. This indicates that bile salt micelles or free bile salts are bound up more
394 effectively into the larger structures. This could have implications for the transport and
395 absorption of lipids and lipophilic compounds if these structures are more stable, and may
396 bind or sequester lipophilic compounds more strongly.

397

398 *3.3. Impact of phytosterols on interfacial tension*

399 The digestibility experiments data presented above show that phytosterols have the
400 capacity ~~of reducing to reduce~~ the rate and extent of lipolysis. The latter process depends on
401 the “quality” of the oil/water interface, i.e., its composition and physico-chemical properties
402 (Reis, Watzke, Leser, Holmberg, & Miller, 2010). **The purpose of the interfacial experiments**

403 was therefore to identify if there were any interfacial mechanisms that would explain the
404 reduction in lipid digestion observed in the presence of phytosterols (Figure 3B). Figure 5A
405 shows that phytosterols were surface active and accumulated at the interface, thus the
406 interfacial tension decreased with increased concentration of phytosterols in the oils.
407 Furthermore, at higher phytosterol concentrations, crystalline structures were observed on the
408 surface of the oil droplets (Figure 5A2), which also affected the shape of the droplets
409 suggesting the formation of a strong, rigid structure at the interface, which could affect lipase
410 accessibility. As anticipated, bile salts alone also reduced the interfacial tension of the oil
411 droplet (~5.5 mN/m), but the ~~presence of~~ phytosterols did not significantly affect the surface
412 tension ~~of when~~ the bile salts ~~were present~~ (Figure 5B). Nevertheless, phytosterols occupied
413 part of the interfacial space, as illustrated by the crystalline structure forming at the edge of
414 the needle tip (red arrow in Figure 5B). **Initially, it was hypothesised that the phytosterols**
415 **reduced the extent of FFA released due to their competition with bile salts at the interface of**
416 **the oil droplets. However, this was not the case as demonstrated by the fairly constant**
417 **interfacial tension overtime (between 5 and 5.5 mN/m).**
418 Immediately after the addition of the enzyme, the interfacial tension dropped further for up to
419 ~35 min when the oil droplet detached from the needle. The bile salts from the aqueous phase
420 seemed to occupy the interface very rapidly (Figure 5B) and must have been more surface
421 active than the phytosterols, consequently the lipolysis process monitored by interfacial
422 tension was unchanged between the oils (Figure 5C). Therefore, ~~in these experiments~~, the
423 phytosterols did not prevent the adsorption of the lipase/colipase onto the surface of the oil
424 droplet. The formation of lipolytic products at the interface, which would have inhibited
425 lipase activity, are likely to be responsible for the reduction in interfacial tension (Reis,
426 Watzke, Leser, Holmberg, & Miller, 2010). Furthermore, Figure 4B2 suggested that the
427 composition of the aqueous phase, in particular the nature of the mixed micelles, may have

428 differed in presence of oil enriched in phytosterols compared with the sunflower oil only.
429 However, the dynamic events taking place at the interface could not be specifically identified
430 with the pendant drop technique. **Indeed, it is likely that the process by which phytosterols**
431 **impact lipolysis is time dependent. The phytosterols may affect the ability of the bile salt**
432 **micelles to remove the lipolytic products because the properties of the micelles change**
433 **following the incorporation of phytosterols. Therefore, these micelles need time to form first**
434 **before any effect of the phytosterols is observed. As a consequence, the kinetic curves**
435 **showed in Figure 3B appeared similar for all samples up to ~6 to 8 min of reaction,**
436 **suggesting that this may be the time required for the changes to the micelle structure to occur**
437 **in the presence of phytosterols.**

438

439 The pH-stat experiments measured the amount of fatty acids released into solution from the
440 oil phase following lipolysis, and **demonstrated** that phytosterols had some impact upon the
441 release **of the FFA**. However, in conjunction with other components, such as polar lipids in
442 the oat oil samples, **phytosterols** could have an additive effect. Furthermore, the more
443 complex interfacial structure and aggregation of the oil bodies would further complicate the
444 digestion process. The micelle behaviour is quite intriguing as this would not be detected by
445 the pH-stat measurements, but could have significant impact on the downstream fate of the
446 mixed micelles / vesicle structures. If the phytosterol were acting to stabilise these structures,
447 **they could reduce both the solubilisation of lipolytic products and also the absorption of**
448 lipids, bile salts and cholesterol, thus helping to explain the mechanisms underpinning the
449 health impact of phytosterols. Further work could focus on the dynamics of these micelles
450 and their bioaccessibility.

451

452 **4. Conclusions**

453 This study evaluated the impact of the composition and overall structure of different
454 lipid systems on the lipolysis process occurring in the duodenal compartment. The findings
455 from the present work revealed that the digestibility of lipids from oat relies on the degree of
456 complexity and/or purity of the oat material. Composition alone is not sufficient to explain
457 the effect that the oat compounds, albeit present in small amount, have on lipid metabolism.
458 Phytosterols appeared to play a role in the reduction in lipid hydrolysis, possibly by affecting
459 the stability and physico-chemical properties of the mixed micelles but the interactions
460 between other oat constituents and digestion agents also seem crucial. The mechanisms
461 responsible for the flocculation of the oil bodies warrant further research. In particular, it
462 would be interesting to investigate the fate of these structures during oral processing and
463 gastric digestion. **Additional work would also focus on the broader matrix effects and**
464 **interactions between oat components that may further explain the complex mechanisms**
465 **underpinning the impact of oat-based products on health.**

466

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473

474 **Conflicts of interest**

475 The authors are not aware of any affiliations, memberships, funding, or financial holdings that
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477

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