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Oat and lipolysis: Food matrix effect

Peter J. Wilde[a], Guadalupe Garcia-Llatas[b], María Jesús Lagarda[b], Richard P. Haslam[c],

Myriam M.L. Grundy[ad*]

[a]Quadram Institute Bioscience, Norwich Research Park, Colney, Norwich NR4 7UA, UK

[b]Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Avda.
Vicente Andrés Estellés s/n, 46100 Burjassot (Valencia), Spain

[c]Rothamsted Research, Department of Plant Sciences, Harpenden, AL5 2JQ, UK

[d]School of Agriculture, Policy and Development, Sustainable Agriculture and Food Systems
Division, University of Reading, Earley Gate, Reading, RG6 6AR, UK

peter.wilde@quadram.ac.uk, guadalupe.garcia@uv.es, m.j.lagarda@uv.es,
richard.haslam@rothamsted.ac.uk, m.m.grundy@reading.ac.uk

*Corresponding author: Myriam M.L. Grundy, School of Agriculture, Policy and
Development, Sustainable Agriculture and Food Systems Division, University of Reading,
Reading, RG6 6AR, UK. Tel.: +44 0 1183 877868, m.m.grundy@reading.ac.uk

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).

1. Introduction

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

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

delivered to the gastrointestinal tract is probably a crucial element to their bioactivity. Indeed,

phytosterol bioavailability and efficacy has been shown to rely on many factors such as the

type and quantity of lipids present, the type of phytosterols (i.e. free, esters or steryl

glycosides sterol, stanol or ester such as steryl glycoside), the source, and the food

microstructure (Ostlund, 2002; Alvarez-Sala et al., 2016; Ferguson, Stojanovski, MacDonald-

Wicks, & Garg, 2016). In plant-based foods, phytosterols are found as a mixture of free and

bound (esters) phytosterols, but not all forms have the same physico-chemical properties and

therefore health benefits (Moreau, Whitaker, & Hicks, 2002). Furthermore, phytosterols are

hydrophobic and poorly soluble in aqueous solutions, so they associate, mainly via

hydrophobic interactions, with various lipophilic structures that are present during digestion,

such as oil droplets, lipid vesicles, membranes, and micelles, and this is thought to be critical

for their functionality (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000; Amiot et al.,

2011).

Some studies have investigated the fate of the phytosterols during *in vitro* lipid

digestion (von Bonsdorff-Nikander et al., 2005; Moran-Valero, Martin, Torrelo, Reglero, &

Torres, 2012; Zhao, Gershkovich, & Wasan, 2012; Alvarez-Sala et al., 2016; Gleize, Nowicki,

Daval, Koutnikova, & Borel, 2016), but mechanistic studies that could provide information

about how they influence lipolysis and micelles formation using different oat matrices are

missing. To improve our understanding gain insight into the role played by the broader oat

matrix composition and structure on lipid digestion, in this work, we examined various

aspects of lipolysis were examined, focusing on the contribution made by phytosterols. We

believe that The kinetics of lipolysis and the mixed micelle formation have important

consequences on lipid and cholesterol uptake. During digestion, complex, dynamic self-

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 (5β-cholestan-3α-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, lysophosphatidylcoline, 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), Δ5-

105 avenasterol (98% purity) and Δ7-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 lysophosphatidylcoline, 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 $CaCl_2$. 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 μL. 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 $CaCl_2$, 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 μM lipase and 75 μM colipase). The

213   initial droplet formed had a volume between 50 and 3 μL 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 their

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

473

474   **Conflicts of interest**

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

476   might be perceived as affecting the objectivity of this work.

477

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