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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

ABSTRACT

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

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

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

Chemical compounds studied in this article

β-sitosterol (PubChem CID: 222284); Campesterol (PubChem CID: 173183); Δ 5-avenasterol (PubChem CID: 5281326); Δ 7-avenasterol (PubChem CID: 12795736); Digalactosyldiacylglycerol (PubChem CID: 25203017); Epicoprostanol (PubChem CID: 91465); Fucosterol (PubChem CID: 5281328); Monogalactosyldiacylglycerol (PubChem CID: 25245664); Phosphatidylcholine (PubChem CID: 6441487); Stigmasterol (PubChem 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 bioactive~~s~~ 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 cholesterol~~s~~ 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 govern~~s~~ the nature and fate

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

2. Materials and Methods

2.1. Materials

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

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

2.2. Material preparation

2.2.1. Oat oil bodies

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

2.2.2. Oils

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

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

2.2.3. Emulsions

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

2.3. Characterisation of the material

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

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

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

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

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

2.6. Interfacial measurements

The interfacial tension at the oil/water interface was measured using the pendant drop technique with a FTA200 pulsating drop tensiometer (FirstTen Angstroms, Portsmouth, VA) as previously described (Chu et al., 2009). An inverted oil drop was formed at the tip of a Teflon-coated J-shaped needle (internal diameter of 0.94 mm) fitted to a syringe with a total volume of 100 µL. The oil drop was formed in a glass cuvette containing 5 mL of 2 mM bis-tris buffer, 0.15 M NaCl, and 0.01 M $CaCl_2$, at pH 7 and maintained at 37°C. The measurements were repeated in presence of bile salts (9.7 mM of mixed NaTC and NaGDC, 53 and 47% respectively) and during lipolysis, in conditions that simulated the physiological environment of the duodenum (9.7 mM bile salts, 15 µM lipase and 75 µM colipase). The initial droplet formed had a volume between 50 and 3 µL depending on the experimental conditions (i.e. amount of phytosterols, presence of bile salts and lipase). The images were captured for 1 h or until the oil drop detached from the tip of the J-shaped needle due to the large decrease in interfacial tension. The shape of the drop in each image was analysed by fitting the experimental drop profile to the Young-Laplace capillarity equation. Each set of experiments was performed in triplicate.

2.7. Microstructural analysis

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

2.8. Statistical analysis

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

3. Results and Discussion

3.1. Characterisation of the oat and sunflower materials

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

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

3.2. Digestibility experiments using the pH-stat method

3.2.1. Lipolysis kinetics

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

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

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

3.3. Impact of phytosterols on interfacial tension

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

was therefore to identify if there were any interfacial mechanisms that would explain the reduction in lipid digestion observed in the presence of phytosterols (Figure 3B). Figure 5A shows that phytosterols were surface active and accumulated at the interface, thus the interfacial tension decreased with increased concentration of phytosterols in the oils. Furthermore, at higher phytosterol concentrations, crystalline structures were observed on the surface of the oil droplets (Figure 5A2), which also affected the shape of the droplets suggesting the formation of a strong, rigid structure at the interface, which could affect lipase accessibility. As anticipated, bile salts alone also reduced the interfacial tension of the oil droplet (~5.5 mN/m), but the ~~presence of~~ phytosterols did not significantly affect the surface tension ~~of~~ when the bile salts were present (Figure 5B). Nevertheless, phytosterols occupied part of the interfacial space, as illustrated by the crystalline structure forming at the edge of the needle tip (red arrow in Figure 5B). Initially, it was hypothesised that the phytosterols reduced the extent of FFA released due to their competition with bile salts at the interface of the oil droplets. However, this was not the case as demonstrated by the fairly constant interfacial tension overtime (between 5 and 5.5 mN/m).

Immediately after the addition of the enzyme, the interfacial tension dropped further for up to ~35 min when the oil droplet detached from the needle. The bile salts from the aqueous phase seemed to occupy the interface very rapidly (Figure 5B) and must have been more surface active than the phytosterols, consequently the lipolysis process monitored by interfacial tension was unchanged between the oils (Figure 5C). Therefore, in these experiments, the phytosterols did not prevent the adsorption of the lipase/colipase onto the surface of the oil droplet. The formation of lipolytic products at the interface, which would have inhibited lipase activity, are likely to be responsible for the reduction in interfacial tension (Reis, Watzke, Leser, Holmberg, & Miller, 2010). Furthermore, Figure 4B2 suggested that the composition of the aqueous phase, in particular the nature of the mixed micelles, may have

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

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

4. Conclusions

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

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Conflicts of interest

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this work.

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