Optimising the ratio of long- to short-chain triglycerides of the lipid phase to enhance physical stability and bioaccessibility of lycopene-loaded beverage emulsions

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Summary

Oil-in-water beverage emulsions (pH3.2) with different long- to short-chain triglyceride (LCT to SCT) ratios were used to encapsulate lycopene. Beverages containing 3% w/w oil from carrier lipids were prepared as follows (w/w): 100:0, 75:25, 50:50, 25:75 and 0:100 (corn oil:tributyrin). The beverages prepared using a low LCT to SCT ratio (0:100) were physically unstable mainly due to Ostwald ripening phenomena, as indicated by confocal laser microscopy. The oil droplet size was significantly reduced for emulsions formulated with corn oil (2.6 μm) compared with tributyrin (5.4 μm). Lycopene was not bioaccessible in beverages formulated with tributyrin only and bioaccessibility increased significantly with increasing the LCT to SCT ratio. Data indicated that bioaccessibility for lycopene is 2.7% for emulsions with high LCT ratios (>75). Results indicate that the carrier lipid phase of emulsion-based systems is critical for the formulation of functional drinks for the delivery of lipophilic bioactive compounds.

Keywords: Beverage emulsion; Lycopene; Corn; Tributyrin; *In vitro* digestion; Bioaccessibility

Introduction

Lycopene is a natural carotenoid pigment responsible for the colour of tomato and other red fruits and vegetables, such as papaya, guava, watermelon and grapefruit (Shi & Maguer, 2000). Lycopene may have a beneficial role in chronic disease prevention, which is mainly attributed to the ability of the carotenoid to inactivate reactive oxygen species and delay oxidative

54 damage. For instance, both *in vitro* and *in vivo* studies, demonstrate its preventive effect against
55 atherosclerosis and other conditions linked to cardiovascular disease (Xaplanteris *et al*., 2012).
56 In addition, lycopene may also be effective for cancer prevention, thanks to its ability to reduce
57 oxidative stress, inhibit cell proliferation and increase apoptosis of human cancer cell lines
58 (Tapiero *et al*., 2004). Epidemiological studies suggest that high plasma levels of lycopene is
59 inversely correlated to the risk of bladder, prostate and breast cancer (Heber & Liu, 2002).
60 While no ideal daily dosage has been established for lycopene, consumption of 10-30 mg of
61 lycopene daily is considered adequate to support optimal health (Devaraj *et al*., 2008).
62 As new scientific evidence emerges supporting the benefits of lycopene for chronic disease
63 prevention, there is increasing consumer interest for foods enriched with this natural pigment.
64 Currently, the main lycopene dietary sources are tomatoes and tomato-based products (i.e.
65 sauces, juice, purees etc.). The bioaccessibility of lycopene from fresh tomato products is
66 considered low (0.1%-3%) due to its lipophilic nature and its subcellular compartmentalisation
67 within chromoplasts (Salvia-Trujillo & McClements, 2016). Furthermore, lycopene contains
68 13 double bonds in its structure and as a result is a highly unsaturated carotenoid, which makes
69 it susceptible to degradation via isomerization and oxidation during processing and storage
70 (Shi, 2000). For these reasons, although the addition of lycopene to food products is strongly
71 desirable, an effective formulation strategy in combination with effective processing methods
72 are required to increase the amount of lycopene released from the food matrix (Reboul *et al*.,
73 2006). Furthermore, a very limited number of studies are available which investigate the
74 bioaccessibility of lycopene powder extract in complex food systems.
75 Emulsion-based systems are becoming increasingly popular food matrices for encapsulating
76 and delivering hydrophobic compounds with health promoting properties. This is mainly due
77 to the fact that lycopene's bioaccessibility significantly improves in the presence of oil, because
78 free fatty acids enhance the solubilisation efficacy of the carotenoid into mixed micelles and

small vesicles during the digestion process (Colle *et al.*, 2012; Yao *et al.*, 2014). Moreover, previous studies indicate that the different length of the fatty acid chain has an impact on the bioaccessibility of lipophilic bioactive compounds. Several *in vitro* studies suggest that the bioaccessibility of hydrophobic components is lower in emulsion-based systems formulated with SCT compared with the ones containing MCT and LCT (Ahmed *et al.*, 2012; Qian *et al.*, 2012; Salvia-Trujillo *et al.*, 2013). The reasons for this outcome may be attributed to the different products formed during lipid digestion. Thus, the formulation and processing steps of the manufacturing process need to be carefully designed to ensure optimum bioavailability of the bioactive ingredient from an emulsion-based system (Raikos & Ranawana, 2017).

The long-term chemical and physical stability of orange oil beverage emulsions formulated with LCT and SCT, which contain lycopene as a bioactive ingredient, has been recently documented (Meroni and Raikos, 2018). The primary focus of the present study was the design of an emulsion-based system that can be used to enhance the bioaccessibility of lycopene and to clarify the specific effect of fatty acid chain length to this respect. Beverage emulsions were prepared from carrier lipids that consisted of different LCT-to-SCT (w/w) mass ratios as follows: 100:0, 75:25, 50:50, 25:75 and 0:100. The effect of different oil composition was evaluated by studying the following parameters: (1) physical stability of freshly prepared beverages monitored by visual observation, Turbiscan and microstructural analysis; and (2) bioaccessibility of lycopene by using an *in vitro* gastro-intestinal digestion model. Results of this study have important implications for developing effective emulsion-delivery systems of lycopene in order to meet consumer demands for food products with health-promoting properties.

Materials and methods

Materials

104 Lycopene powder (lycopene>10%, redivivo®) was kindly provided by DSM Nutritional
105 Products Ltd (Heanor, UK). Tocopherol-stripped corn oil (LCT) and tributyrin (SCT) were
106 purchased from Sigma–Aldrich (Dorset, UK). Citric acid, amylase (type VI-B), pepsin,
107 pancreatin, bile extract and Nile Red were purchased from Sigma Aldrich (Dorset, UK). Pure
108 Whey Isolate^{TM} 97 powder (WPI, 97% protein) was purchased from Bulk Powders (Colchester,
109 UK). All other reagents used were of analytical grade.
110
111 Determination of Total Lipids as Fatty Acid Methyl Esters (FAME)
112 The fatty acid composition is determined as the methyl esters of fatty acids by a Hewlett
113 Packard 6890 gas-liquid chromatograph (Avondale, PA) equipped with a 50 m × 20 mm
114 Chrompac CP7488 CP Sil-88 capillary column (film thickness 0.20 μm). The experimental
115 procedure is described in detail in literature published previously (Meroni & Raikos, 2018).
116 Separation was recorded with HP GC Chemstation software (Hewlett Packard, Avondale, PA).
117 Results are expressed as % of total fatty acids.
118
119 Preparation of oil-in-water (o/w) beverage emulsions
120 Emulsion beverages were obtained by mixing the following ingredients using a standardized
121 (w/w) recipe: 90.3% water, 3% WPI, 3% oil, 0.7% citric acid, 1% lycopene powder. To
122 investigate the effect of fatty acid chain length, emulsions were prepared from carrier lipids
123 that consisted of different mass ratios of LCT-to-SCT (w/w) as follows: 100:0, 75:25, 50:50,
124 25:75 and 0:100. A coarse emulsion was initially formed by slowly adding oil to the water
125 phase and mixing the rest of the ingredients using an ultra-compact digital mixer system (Cole-
126 Palmer, Cambridgeshire, UK) for 5 min at 1000 rpm. Emulsions were formed by passing the
127 coarse emulsions twice through a single stage valve homogenizer (APV-1000, SPX Flow
128 Technology, West Sussex, UK) at 50 MPa. The process was repeated twice to generate two

129 different batches for every oil type beverage. Physical stability analysis was performed
130 immediately after emulsion formation. For other analyses samples were stored at 4 ºC for up
131 to one week after emulsion formation. The method of preparation is described in detail in
132 previous work (Meroni & Raikos, 2018).
133
134 Emulsion physical stability
135 A Multiple Light Scattering method (Mengual *et al*., 1999) was employed to assess the physical
136 stability of beverage emulsions using a Turbiscan MA2000 (Formulaction, Ramonville St.
137 Agne, France). The apparatus can measure backscattered light as a function of the distance
138 along the axis of the sample container and time by using a synchronous optical sensor that
139 receives light backscattered by the sample. Backscattered flux is directly related to the photon
140 transport mean free path (l*).
141 $$BS = \frac{1}{\sqrt{l*}} \quad (1)$$
142 Mie theory states that l^* is inversely proportional to the volume fraction of samples and
143 proportional to the mean diameter d, which is represented by the equation:
144 $$l^*(\Phi,d) = \frac{2d}{3\Phi(1-g)Q_s} \quad (2)$$
145 where d is the average particle diameter, Φ is the volume fraction occupied by particles, g is
146 the asymmetry factor and Qs is the extinction cross-section divided by the geometrical cross-
147 section. Quantification of creaming was enabled by calculating the particle migration velocity
148 and the thickness of the cream phase. A series of scans was repeated for the beverages at 5 min
149 intervals from top to bottom and the intensity of light backscattered or transmitted during a 1
150 h period at 37°C was recorded. The refractive indices of the dispersed and continuous phase
151 which were used to compute the mean spherical equivalent diameter were 1.45 and 1.33
152 respectively. Turbisoft Lab 2.2 software was used to monitor the destabilization phenomena
153 through the variation of the backscattering flux over time.

154

155 Transparency analysis

156 Transparency of lycopene beverage emulsions was acquired by measuring the absorbance of
157 diluted beverage samples (x200) at 600 nm by means of A Pye Unicam UV-4 UV-VIS scanning
158 spectrophotometer (Spectronic Camspec Ltd, Leeds, UK) according to the method described
159 by Ha *et al*. (2015). The following equation was used to calculate transparency:

160 $$T = \frac{1}{10^{A}} \quad (3)$$

161 where T is the transparency and A is the value of absorbance at 600 nm.

162

163 Emulsion microstructure

164 Emulsion microstructure was analyzed by means of confocal laser scanning microscopy
165 (CLSM) using a Carl Zeiss LSM 710 (Carl Zeiss Ltd, Cambridge, UK) according to the method
166 of Chevallier *et al.* (2016). In brief, 10 µl Nile Red solution (0.125% w/v in propylene glycol)
167 were mixed with 1 ml of emulsion to dye the fat globules. Samples were kept at 25 °C in the
168 dark for 15 min, then 50µl of each emulsion were placed on microscope cover glass.
169 Observations were conducted at excitation wavelength of 543 nm for Nile Red dye and using
170 a 63x oil immersion objective. Images for each sample were captured by scanning at a
171 resolution of 1024x1024 pixels.

172

173 *In vitro* gastrointestinal digestion method (IGD)

174 The method described by Minekus *et al.* (2014) based on the standardized static *in vitro*
175 digestion model suitable for food was used to determine lycopene bioaccessibility with several
176 modifications (Meroni & Raikos, 2018). The compositions of the simulated digestion fluids for
177 the oral, gastric and intestinal phases are presented in Table 1. Four independent IGD runs were
178 performed to obtain replicates and assess reproducibility of results.

179

180 Quantification of lycopene

181 Lycopene in beverages and digested samples was quantified by means of a reverse phase HPLC
182 method using fluorescence and UV-visible detection according to the method of Hess *et al.*
183 (1991) with modifications (Meroni & Raikos, 2018). Peaks were identified by comparing the
184 retention times and UV-Vis spectral data with those of the corresponding standards and
185 quantification was enabled by using the standard curves. Measurements were determined with
186 mixed standards containing carotenoids and tocopherols at appropriate concentrations and
187 results were expressed in μg/g of oil. Echinone was added as an internal standard for accurate
188 quantitative measurement.

189

190 Bioaccessibility determination

191 Following the digestion protocol, the digesta was centrifuged (3220 x g) at 25 °C for 40 min
192 using a MiniSpin® plus centrifuge (Fisher Scientific UK, Loughborough, UK). The middle
193 phase of the centrifuged sample was assumed to contain solubilized lycopene in mixed micelles
194 (Ha *et al*., 2015). Aliquots were collected directly from raw digesta and from the middle phase
195 of centrifuged samples and were prepared for RP-HPLC analysis for lycopene quantification.
196 The following equation was used to determine bioaccessibility of lycopene:

197 Lycopene bioaccessibility (%) = 100 x $\frac{C_{micelle}}{C_{initial\ sample}}$ (4)

198 where $C_{micelle}$ is the lycopene concentration in the mixed micelle phase after *in vitro* digestion.

199

200 Statistical analysis

201 All experiments were conducted on at least two freshly prepared beverages. Results are
202 expressed as means ± standard deviation (SD) of at least three replicates. Data were subjected
203 to statistical analysis by SPSS Statistics 22 software. Means were analyzed by analysis of

variance (ANOVA) and significant differences ($p<0.05$) were detected by the *Scheffé's* post hoc test.

Results and discussion

Effect of carrier oil on physical stability and optical properties of beverage emulsions

A series of oil-in-water emulsions was produced by high pressure homogenization that contained different ratios of LCT (corn oil) and SCT (tributyrin). Tributyrin (SCT), is a common food additive composed of butyric acid and is naturally present in butter. Corn oil (LCT) contains a desirable fatty acid profile and is a key ingredient in many processed foods. The detailed fatty acid composition of each carrier oil is in agreement with published data (Table 2). As expected the analysis of the oils confirmed that corn oil is a good source of long chain triglycerides, predominantly linoleic acid, oleic acid and palmitic acid. Tributyrin is a saturated short chain triglyceride composed of butyric acid esterified to glycerol.

The physical stability of the beverage emulsions was monitored by a. visual observation, b. Turbiscan analysis and c. microstructural analysis. All methods employed in this study, indicated that the fatty acid composition had a major impact on the beverage emulsions' stability and their susceptibility to instability phenomena. Figure 1 shows the beverages standing at room temperature for 24 h. Visual observation of the samples suggested that beverages with a high corn oil concentration were prone to creaming as evidenced by the formation of a cream layer at the top of the emulsion. On the other hand, beverages containing 100% tributyrin were prone to sedimentation phenomena with the droplets being visually detected at the bottom of the emulsion. These differences in the direction of the migration pattern between samples with high levels of corn oil and tributyrin are governed by their corresponding densities (0.91 g/ml & 1.03 g/ml at 25 ºC respectively).

228 A more detailed insight into the physical stability of the beverage emulsions is given by
229 Turbiscan analysis, which enables the early detection of instability phenomena by evaluating
230 the optical transmission and backscattering profiles of undiluted emulsion samples. Results
231 obtained from Turbiscan analysis are presented in Table 3. Increasing the LCT/SCT ratio of
232 the beverage led to significant ($p<0.05$) reduction of the particle size. The beverages formulated
233 with tributyrin (100%) have an average particle size droplet more than twice as large compared
234 with samples formulated with corn oil (100%). Results are attributed to Ostwald ripening (OR)
235 phenomena which are commonly observed in food systems containing emulsified tributyrin
236 (Wooster *et al*., 2008). Ostwald ripening is the process by which the components of the
237 discontinuous phase diffuse from small to large droplets through the continuous aqueous phase
238 (Kabalnov & Shchulkin, 1992). Data from microstructural analysis of the beverages also
239 indicates that OR is the main cause of instability for the samples formulated with 100%
240 tributyrin (Figure 2). Ostwald ripening rates are directly proportional to oil molar volume
241 (Wooster *et al*., 2008). Emulsions formulated with tributyrin as the sole lipid phase are highly
242 unstable to droplet growth due to Ostwald ripening (OR) because of the relatively high water
243 solubility of this low molecular weight triacylglycerol (Li *et al*., 2009). The addition of corn
244 oil enhances the physical stability of the beverages by significantly reducing the average
245 droplet particle size and this effect is more profound with increasing the LCT ratio of the
246 mixture (Figure 2). Previous research has indicated that droplet enlargement due to OR can be
247 greatly reduced by the addition of highly hydrophobic triglycerides such as corn oil
248 (McClements *et al*., 2012). The enhanced stability against OR when LCT are added to
249 tributyrin is attributed to the altered lipid phase composition between differently size droplets,
250 a phenomenon known as compositional ripening. The large triacylglycerol molecules of corn
251 oil with low water-solubility prevents droplet growth by a ripening effect that opposes OR
252 effect (Kabalnov & Shchulkin, 1992). On the other hand creaming rates are higher for samples

253 with a high LCT content, which is in agreement with the visual observations of the beverage
254 emulsions during storage at 25 ºC for 24 h. Thus, although corn oil is essential to inhibit OR
255 and stabilize beverage emulsions which contain SCT, it is not ideal for formulations as the sole
256 lipid carrier due to creaming effects. Results suggest that a combination of corn oil and
257 tribyturin (75:25) is desirable to obtain favorable physical stability of beverage emulsions.
258 Optical properties of emulsion-based products are of paramount importance and relate to
259 consumer liking and product acceptability. The interplay between nutrient composition,
260 emulsion microstructure and appearance needs to be elucidated for designing emulsion-based
261 products with desirable characteristics. The appearance of emulsions to the human eye is
262 determined by interactions with electromagnetic radiation in the visible region of the spectrum.
263 The colour of an emulsion is determined by the presence of chromophoric compounds. Light
264 scattering effects are primarily dependent by the characteristics of emulsion droplets such as
265 size, concentration, and refractive index (Park *et al*., 2013). Figure 3 shows the relationship
266 between particle size, LCT-SCT ratio and (%) transparency. Results indicated that increasing
267 particle size increased transparency. The findings of this study are not in agreement with
268 previous research which suggests that small particle size corresponds to higher transparency of
269 nanoemulsions (Ha *et al*., 2012). At nanoscale the optical properties are largely influenced by
270 the particle size of the dispersed particles. For macroemulsions, the refractive indices of the oil
271 and water phase play a major role in emulsion transparency. If both phases have the same or
272 similar refractive index (n), there will be neither reflection nor refraction and the system will
273 appear homogeneous and entirely transparent (Poras *et al*., 2008). In the present study, the
274 refractive index difference between oil and aqueous phase is higher for beverages with
275 increasing LCT (water: 1.33, corn: 1.47, tributyrin: 1.43), which seems to be the main reason
276 for the improved transparency shown by the beverages with a high SCT/LCT ratio.
277

278 Impact of carrier oil on bioaccessibility of lycopene during *in vitro* gastric digestion of
279 beverage emulsions
280 The term bioaccessibility refers to the fraction of a nutrient of compound that can be absorbed
281 in the small intestine after it has been released from the food matrix during digestion. A number
282 of studies indicate that the addition of dietary fat is beneficial for increasing the bioaccessibility
283 of carotenoids (Brown *et al*., 2004; González-Casado *et al*., 2018). In this study, five ratios
284 from two oil types were chosen to assess the effect of fatty acid length and saturation degree
285 on lycopene bioaccessibility from a beverage emulsion using a simulated gastrointestinal
286 digestion procedure. Bioaccessibility was determined by taking into account the lycopene
287 concentration in the mixed micelle phase and in the undigested sample. This calculation
288 method quantifies the lycopene fraction which is made available for absorption in relation to
289 the amount originally present in the beverage, and is thus more indicative of the amount of the
290 carotenoid lost during the digestion process. Results clearly indicated that lycopene
291 bioaccessibility was significantly affected by the type of carrier oil used for emulsion
292 formulation (Fig. 4). In particular, the highest bioaccessibility value for lycopene was recorded
293 for the beverage formulated with an LCT-to-SCT ratio of 75:25, whereas no lycopene was
294 available for absorption from the samples formulated with tributyrin only. In fact, lycopene
295 bioaccessibility increased with increasing the amount of LCT present in the oil phase of the
296 emulsion. The present findings are in agreement with previously published data, which further
297 confirm the hypothesis that bioaccessibility of carotenoids depends on the type of carrier oil
298 used for emulsion formulation. In a similar study, bioaccessibility of β-carotene encapsulated
299 in nanoemulsions was highly affected by the carrier oil type and decreased in the order
300 LCT>MCT>undigested oil (Qian *et al*., 2012). Moreover, Salvia-Trujillo *et al*. (2013) reported
301 that β-carotene bioaccessibility in edible nanoemulsions increased from 14% to 86% as the
302 LCT fraction of the oil phase increased from 0% to 100%. Similarly, emulsions containing

303 MCT or LCT within the carrier lipid were able to substantially increase the bioaccessibility of
304 curcumin, a pigment from turmeric, which can be attributed to their ability to form mixed
305 micelles capable of solubilizing components of lipophilic nature (Ahmed *et al*., 2012). LCT
306 from corn oil are capable of forming mixed micelles with a large hydrophobic core, whereas
307 those produced by digestion of SCT, do not form mixed micelles that are capable of
308 solubilizing large, hydrophobic carotenoids (Fatouros & Mullertz, 2008). Thereby, the
309 colloidal structures formed by the digestion of LCT allow the accommodation of lycopene,
310 which in turn increases the solubilisation capacity of the carotenoid prior to absorption. This
311 mechanism is proposed in several papers (Rao *et al*., 2013; Qian *et al*., 2012; Salvia-Trujillo *et*
312 *al*., 2013) and may explain why lycopene bioaccessibility was significantly higher for
313 beverages with increasing LCT-to-SCT ratios. However, this hypothesis needs to be further
314 investigated to confirm whether LCT can enhance the absorption of lycopene *in vivo*.

315 An additional factor that has been previously reported to affect lycopene bioaccessibility is
316 the size of the fat globules present in the emulsions. Emulsions with small droplets have large
317 specific surface area that increases the accessibility to lipases, co-lipases, and endogenous
318 surfactants (bile salt, cholesterol, phospholipids); consequently the digestion rate,
319 solubilisation efficiency and the absorption rate of the encapsulated compound is enhanced by
320 reducing the droplet size. The first study to report the direct correlation between oil droplet size
321 and bioaccessibility of lycopene encapsulated in oil-in-water nanoemulsions was published by
322 Ha *et al*. (2015). A further study showed that the droplet size of excipient emulsions can
323 determine the bioaccessibility of lycopene from tomato juice (Salvia-Trujillo & McClements,
324 2016). In this study, the bioaccessibility of lycopene increased from 10% to 12.5% and this
325 effect was attributed to the reduction of size of the oil globules. This resulted in higher exposure
326 of lipid surface area, which in turn enhanced the digestion rate and efficiency by digestive
327 enzymes. Salvia-Trujillo *et al*., 2013). These results are in agreement with the findings of the

present study, which show an inverse correlation between droplet size and bioaccessibility; the highest lycopene bioaccessibility (2.7%) was obtained for the beverages with the highest LCT-to-SCT ratio (100:0) and the smallest droplet size (2.6μm±0.1). These results are consistent with the previously reported data (Ha *et al*., 2015; Salvia-Trujillo & McClements, 2016; Salvia-Trujillo *et al*., 2017), and confirm the importance of droplet size for the bioaccessibility and absorption of lycopene from emulsion-based systems.

Conclusions

This study shows that the ratio of long- to short-chain triglycerides of the dispersed oil phase has a significant impact on the physical stability of beverage emulsions. The addition of corn oil significantly reduced the average droplet size and enhanced the physical stability of the beverages by retarding Ostwald ripening phenomena; this effect was more profound with increasing the LCT ratio of the mixture. Nevertheless, emulsions with high LCT content had higher creaming rates and lower transparency compared to the beverages with high SCT-to-LCT ratios. The bioaccessibility of lycopene was significantly increased with the inclusion of LCT in the beverage formulation, whereas it was not bioaccessible in beverages containing tribyturin only. Both droplet size and the solubilisation efficiency of LCT's impact on lycopene bioaccessibility from beverage emulsions.

Considering the results obtained in this study, we can conclude that the most favorable formulation for beverage emulsions containing lycopene should contain a mixture of LCT and SCT, at a ratio 75:25. Findings of this research highlight the importance of the selection of carrier oil for designing emulsion delivery systems capable of encapsulating lycopene. Further improvements with respect to lycopene formulation in complex food systems are required and future studies to clarify the effect of different oil types on the bioavailability of lycopene using *in vivo* models (cells, animals or humans) are needed.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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456

457 **Legends to Figure**

458 **Fig. 1**. Effect of LCT to SCT ratio on the particle migration pattern by visual observation at
459 room temperature for 24 hr.
460 **Fig. 2**. Confocal Laser Scanning Microscopic (CLSM) images of beverage emulsions with
461 different LCT to SCT ratios. Lipid droplets are stained with Nile red and scale is scale bar
462 equals to 10 μm.
463 **Fig. 3**. Relationship between transparency (%), LCT to SCT ratio and particle size (μm) of
464 lycopene beverage emulsions.
465 **Fig. 4**. Bioaccessibility (%) of lycopene beverage emulsions formulated with different LCT to
466 SCT ratios. The centrifuged fraction of the raw digesta (mixed micelles) of the beverage
467 samples is shown in the picture. Different letters denote significant differences ($P < 0.05$)
468 between samples.
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