Food & Function

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Journal:	*Food & Function*
Manuscript ID:	FO-ART-08-2014-000725.R1
Article Type:	Paper
Date Submitted by the Author:	16-Sep-2014

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Enhancing Vitamin E bioaccessibility: Factors impacting solubilization and hydrolysis of α-tocopherol acetate encapsulated in emulsion-based delivery systems

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Journal: Food and Function

Submitted: August, 2014

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1 ## Abstract

2 Oil-soluble vitamins are often encapsulated within emulsion-based delivery systems to facilitate
3 their incorporation into aqueous-based products. We have examined the influence of carrier oil type
4 and simulated small intestinal fluid (SSIF) composition on the bioaccessibility of emulsified vitamin
5 E using a gastrointestinal model. Oil-in-water emulsions containing vitamin E acetate were
6 prepared using bile salts as emulsifier, and either long chain triacylglycerols (glyceryl trioleate, LCT)
7 or medium chain triacylglycerols (glyceryl trioctanoate, MCT) as carrier oils. The addition of
8 calcium ($CaCl_2$) to the SSIF increased the extent of lipid digestion in LCT-emulsions, but had little
9 impact in MCT-emulsions. The bioaccessibility of vitamin E increased in the presence of calcium
10 and phospholipids (DOPC) in LCT-emulsions, but decreased in MCT-emulsions. The highest
11 bioaccessibility (≈ 66%) was achieved for LCT-emulsions when the SSIF contained both calcium
12 and phospholipids. The conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion
13 was considerably higher for LCT-emulsions when calcium ions were present in the SSIF, but was not
14 strongly affected by SSIF composition for MCT-emulsions. In general, this research provides
15 important information about the factors influencing the bioaccessibility of emulsified vitamin E,
16 which could be used to design more effective emulsion-based delivery systems for increasing the
17 oral bioavailability of this important bioactive component.

18 **Key words:** vitamin E acetate; vitamin E; α-tocopherol; digestion; bioaccessibility;
19 bioavailability; bile salts; emulsion; nanoemulsion; calcium; phospholipid; lipase.

20

21 ## 1. Introduction

22 The term "vitamin E" refers to a group of naturally occurring compounds that have common
23 molecular, physicochemical, and biological features, with α-tocopherol being the most biological
24 active form [1]. As well as its important role as an essential nutrient, vitamin E may also provide
25 additional health benefits, such as reducing cardiovascular disease, diabetes, and cancer due to its
26 antioxidant and non-antioxidant biological activities [2-4]. Vitamin E can also protect lipids in foods
27 against oxidation due to the ability of α-tocopherol to trap peroxyl radicals, which are responsible for
28 the initiation of lipid oxidation [5, 6]. The food industry is therefore interested in fortifying functional
29 foods and beverages with vitamin E due to its antioxidant activity and potential health benefits [7].
30 However, the incorporation of α-tocopherol into many commercial products is a challenge because of
31 its relatively low chemical stability, water-solubility, and bioavailability [2-4].
32 Emulsion-based delivery systems are especially suitable for encapsulating, protecting and
33 delivering lipophilic bioactive components, such as ω-3 fatty acids, carotenoids, curcuminoids
34 phytosterols, and oil-soluble vitamins [7-9]. A considerable amount of research has already been
35 carried out to identify the major factors affecting the bioavailability of lipophilic bioactive molecules
36 encapsulated within emulsion-based delivery systems, such as particle size, physical state, and
37 interfacial properties [10-13]. It is important that any encapsulated bioactive component has a high oral
38 bioavailability so that it can effectively deliver its health benefits after ingestion. However, a
39 number of physicochemical and physiological processes occur within the human gastrointestinal tract
40 that impact the oral bioavailability of lipophilic vitamins [14, 15]. After ingestion, vitamin E is usually
41 released from the food matrix, solubilized within mixed micelles in the small intestine, and then
42 transported to the epithelium cells where it is absorbed [16, 17]. Mixed micelles are complex colloidal
43 structures formed from bile salts and phospholipids present in the intestinal fluids, as well as free
44 fatty acids and monoacylglycerols generated by lipid hydrolysis. The bioaccessibility of oil-soluble
45 nutraceuticals, vitamins and drugs has previously been shown to increase when the amount of mixed
46 micelles present within the intestinal fluids increases, which typically occurs as the amount of
47 co-ingested digestible lipids (triacylglycerol hydrolysis products) increases [18-20]. The bioaccessibility
48 also depends on the nature of the mixed micelles formed after lipid digestion, *i.e.,* the composition
49 and nature of the colloidal structures formed [19, 21-23]. Indeed, highly lipophilic components

50 encapsulated using delivery systems containing long chain triglycerides (LCT) have been reported to
51 have a higher bioaccessibility than those containing medium chain triglycerides (MCT), which can
52 be attributed to the ability of the mixed micelles formed by LCT to incorporate larger lipophilic
53 molecules [24, 25].
54 The chemical form of the vitamin E present within a food or beverage product also influences its
55 bioavailability. Vitamin E is often incorporated into foods in an esterified form (α-tocopherol
56 acetate) because it has a higher chemical stability than the non-esterified form (α-tocopherol) [26].
57 However, the esterified form of vitamin E has a lower bioaccessibility than the free form,
58 presumably because it is more difficult to incorporate into mixed micelles [27-30]. Consequently, the
59 bioavailability of vitamin E would be increased if there were greater conversion of α-tocopherol
60 acetate to α-tocopherol in the gastrointestinal tract due to the presence of digestive enzymes.
61 Numerous factors influence the digestion of emulsified triglycerides by pancreatic lipase and the
62 subsequent formation of mixed micelles, including droplet surface area, interfacial composition,
63 carrier oil type, calcium ions, bile salts, and phospholipids [31-33]. Calcium ions have been identified
64 as playing a particularly important role in the digestion of emulsified LCTs. In the absence of
65 calcium, long-chain free fatty acids (FFAs) accumulate at the oil-water interface and inhibit further
66 lipid digestion, presumably by preventing lipase from reaching non-digested lipids at the core of the
67 emulsion droplets [34]. The presence of calcium ions facilitates lipid digestion due to the formation of
68 insoluble calcium soaps that remove long-chain FFAs from the emulsion droplet surfaces, thereby
69 allowing the lipase to remain in close contact with the non-digested lipids [35-40]. One might expect
70 the bioaccessibility of oil-soluble vitamins to increase in the presence of calcium ions since then
71 more LCTs would be digested, leading to the release of more vitamin molecules from the lipid
72 droplets and to the formation of more mixed micelles capable of solubilizing them. On the other
73 hand, calcium ions may interact with mixed micelles and form insoluble complexes that actually
74 reduce the bioaccessibility of oil-soluble vitamins by preventing them from being absorbed [41]. The
75 rate and extent of lipid digestion in emulsion-based delivery systems comprised of MCT (rather than
76 LCT) have been shown to be much less sensitive to calcium ions [32]. Research is therefore needed to
77 determine the potential effect of carrier oil type and calcium ions on the bioaccessibility of
78 emulsified oil-soluble vitamins.
79 In a recent study using a simulated gastrointestinal tract (GIT) model, we found that the

80 bioaccessibility of vitamin E was higher in emulsions prepared using LCTs than in those prepared
81 using MCTs [24]. The LCT (corn oil) and MCT used in that study were food-grade oils containing a
82 mixture of different triacylglycerols. In a follow up study, we used model mixed micelles
83 assembled from well-defined fatty acids (C8:0 or C18:1) (**Figure 1**) to provide further insights into
84 the impact of MCT and LCT digestion products on vitamin E solubilization [42]. However, we did not
85 find an appreciable difference between the vitamin E solubilization capacity of mixed micelles
86 prepared from C8:0 or C18:1. The apparent discrepancy between these two studies may have been
87 due to differences in the nature of the lipids used or due to differences in the simulated
88 gastrointestinal conditions used. For example, mixed micelles were formed by digesting food-grade
89 MCTs and LCTs in simulated intestinal fluids in the initial study, resulting in a complex mixture of
90 free fatty acids and monoacylglycerols, which would interact with the bile extract. However, the
91 mixed micelles were formed by simply mixing pure free fatty acids (C8:0 or C18:1) with pure bile
92 salts (sodium cholate and sodium deoxycholate) **(Figure 1)** in the follow up study.
93 The purpose of the current study was therefore to use a simulated GIT model to establish the
94 influences of carrier oil type (C8:0 *versus* C18:1) and small intestinal composition (bile type, calcium,
95 and phospholipids) on the bioaccessibility of emulsified vitamin E (**Figure 1**). The information
96 gained from this study will be useful for designing more effective emulsion-based delivery systems
97 for these important lipophilic bioactive components.

98 2. Materials and methods

99 2.1 Materials

100 Sodium cholate (NaC), sodium deoxycholate (NaDC), glyceryl trioleate, and glyceryl
101 trioctanoate were purchased from the Sigma Chemical Company (St. Louis, MO). 1,
102 2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids, Inc.
103 (Alabaster, AL). Vitamin E acetate was kindly supplied by BASF (Florham Park, NJ). Lipase
104 from porcine pancreas pancreatin (activity >2.0 USP units/mg, Type II, L3126, Batch # SLBC9250V)
105 was obtained from the Sigma Chemical Company (St. Louis, MO). Here, 1 USP unit will
106 hydrolyze 1.0 microequivalent of fatty acid from triacetin in 1 hr at pH 7.4 at 37°C. Bile extract
107 (B8631, Batch # MKBQ8333V) was also obtained from the Sigma Chemical Company (St. Louis,
108 MO). This material was reported to contain glycine and taurine conjugates of hyodeoxycholic acid

109 and other bile salts. All other chemicals used were of analytical grade. Double distilled water was
110 used for the preparation of all solutions and emulsions.
111

112 ## 2.2 Vitamin E emulsion preparation

113 Vitamin E emulsions were prepared by homogenizing 2.5 wt% lipid phase (Vitamin E acetate :
114 triacylglycerol = 1:1) with 97.5 wt% aqueous phase. The aqueous phase was comprised of
115 surfactant (0.5 wt% bile salt) and buffer solution phosphate-buffered saline, pH 7.0). A coarse
116 emulsion premix was prepared by blending the lipid and aqueous phases together using a high-speed
117 mixer (Bamix, Biospec Products, Bartlesville, OK) for 2 min at room temperature. Fine vitamin E
118 emulsions were formed by passing the coarse emulsions through an air-driven microfluidizer
119 (Microfluidics, Newton, MA, USA). The coarse emulsions were passed through the homogenizer
120 for 4 passes at 9,000 psi. The resulting systems were designed to represent the emulsified lipids
121 that are present within the small intestine after ingestion of fatty foods, which typically consist of
122 lipid droplets coated by bile salts (since these biological surfactants typically displace the original
123 surfactants from the lipid droplet surfaces in the gastrointestinal tract).

124 ## 2.3 Particle characterization

125 Mean particle sizes and particle size distributions of initial emulsions and samples exposed to
126 GIT conditions were measured using static light scattering (Mastersizer 2000, Malvern Instruments,
127 Malvern, UK), while their electrical charge (ζ-potential) was measuring by electrophoretic mobility
128 (Nano-ZS, Malvern Instruments, Worcestershire, UK). The mean particle diameter, particle size
129 distribution, and electrical charge of mixed micelles were determined by dynamic light scattering and
130 electrophoretic mobility (Nano-ZS, Malvern Instruments, Malvern, UK). Samples were
131 equilibrated for 1 min inside the instrument before data were collected over at least 10 sequential
132 readings and analyzed using the Smoluchowski model.

133 ## 2.4. *In vitro* small intestine digestion

134 Samples (10 ml) were added to a clean beaker, mixed with 20 mL phosphate-buffered saline
135 (PBS, 10 mM, pH 7.0), incubated in a water bath (37 ºC) for 10 min, and then adjusted to pH 7.0
136 using NaOH solution (range from 0.05 to 1 M). The mixture was then incubated for 2 h at 37 ºC
137 with simulated small intestinal fluids (SSIF) of different compositions (**Table 1**). SSIFs with five

138 different compositions were prepared: bile extract without $CaCl_2$; bile extract with $CaCl_2$; bile salts
139 (NaC and NaDC) without $CaCl_2$; bile salts (NaC and NaDC) with $CaCl_2$; bile salts (NaC and NaDC)
140 with $CaCl_2$ and DOPC. A pH-stat (Metrohm, USA Inc.) was used to monitor and control the pH (at
141 pH 7.0) of the digestion solution after the sample and SSIF were mixed [32]. The amount of alkali
142 solution (0.25 M NaOH) that had to be added to the reaction chamber to maintain the pH at 7.0 was
143 recorded, and used to determine the percentage of free fatty acids (FFA) released from the system
144 (McClements & Li, 2010a). A control (containing bile salts but no oil) was run under the same
145 conditions as the samples, and the amount of alkali titrated into the reaction chamber for the control
146 was subtracted from that for the samples before calculating the FFA released. Samples were also
147 taken for physicochemical and structural characterization after 2 h incubation in the small intestinal
148 stage.
149 In this study, each sample was only tested using a simulated small intestine model so that we
150 could focus on the physicochemical events occurring in this region of the gastrointestinal tract (GIT),
151 without having to consider structural or compositional changes occurring in the mouth or stomach
152 phases, which would have complicated interpretation of the results. Nevertheless, it would be
153 useful in future studies to pass samples through a full GIT model (mouth, stomach, and small
154 intestine) to more accurately represent the changes occurring in the human GIT.

155 **2.5. Bioaccessibility determination**

156 Vitamin E is solubilized within mixed micelles consisting present in the intestinal lumen before
157 uptake into intestinal epithelial cells [43]. The fraction of lipophilic bioactive compounds solubilized
158 within the mixed micelle phase is usually regarded as the bioaccessibility [44, 45]. The bioaccessibility
159 of vitamin E was determined using a method described previously [24]. Briefly, the digesta resulting
160 from small intestine digestion of the samples was collected and then centrifuged (4000 rpm; CL10
161 centrifuge, Thermo Scientific, Waltham, MA, USA) at 25 ºC for 40 min. Samples after
162 centrifugation separated into an optically opaque sediment phase at the bottom, a relatively clear
163 aqueous phase in the middle, and sometimes a thin oily or creamed phase at the top. The middle
164 phase was assumed to be the “micelle” phase that solubilized the vitamin E. Vitamin E was
165 extracted from the middle phase using an organic solvent mixture (1:3 isopropanol and isooctane) at
166 1:5 and then centrifuged at 1750 rpm for another 10 min. One mL of the top layer was removed

167 and dried using nitrogen evaporation and stored in the -80 ºC refrigerator prior to further analysis.
168 Before detection by HPLC, samples were dissolved in 200 µL methanol. The vitamin E
169 concentrations in the samples were determined using HPLC (Shimadzu, Kyoto, Japan). A C_{18} reverse
170 phase column (150 - 4.6 mm, 5 µm, Beckman Coulter) was used for the chromatographic separation
171 of α-tocopherol acetate and α-tocopherol. The flow rate of the mobile phase was 1.0 ml/min. An
172 isocratic elution was carried out using HPLC-grade solvent (95% methanol and 5% double distilled
173 water containing 0.5 % phosphoric acid). The α-tocopherol acetate and α-tocopherol contents were
174 determined using a PDA detector at 295 nm. Tocopherol quantification was determined using
175 external standards. The overall bioaccessibility of vitamin E was estimated using the following
176 expression:

$$Bioaccessibility = \frac{C_{micelle}}{C_{Total}} \times 100\%$$

177

178 Here $C_{micelle}$ and C_{Total} represent the total concentration of vitamin E (α-tocopherol acetate +
179 α-tocopherol) in the micelle phase and in the overall system after digestion, respectively. The
180 percentage of specific forms of vitamin E solubilized within the micelle phase was also calculated
181 using the same expression, but for α-tocopherol acetate and for a-tocopherol separately. The
182 conversion of α-tocopherol acetate to α-tocopherol in the overall system after digestion was
183 calculated from the following expression:

184 $$Conversion = \frac{C_{VE}}{C_{Total}} \times 100\%$$

185 Here C_{VE} and C_{Total} represent the concentration of α-tocopherol and the total concentration of vitamin
186 E (α-tocopherol acetate + α-tocopherol) in the overall system after digestion, respectively. A
187 preliminary experiment was carried out to estimate the recovery of the total tocopherols using the
188 solvent extraction and HPLC analysis method described above. The recovery of the total tocopherols
189 (VE + VE acetate) was always > 90%, which indicates that the methods used were appropriate.

190 ## 2.8 Statistical analysis

191 All measurements were performed on at least two freshly prepared samples (*i.e.,* new samples
192 were prepared for each series of experiments), and two separate measurements were made per
193 sample, leading to four measurements in total. The means and standard deviations were calculated

194 from this data. Statistical differences were performed by ANOVA analysis.

195 3. Results and discussion

196 3.1 Impact of SSIF composition and carrier oil on gastrointestinal fate of emulsions

197 Initially, we studied the influence of SSIF composition and carrier oil type on the potential
198 gastrointestinal fate of emulsion-based delivery systems using a simulated small intestine model
199 (pH-stat). Vitamin-fortified emulsions containing 2.5 wt% lipid phase were produced using either
200 long chain triglycerides (C18:1) or medium chain triglycerides (C8:0) as carrier oil. The emulsions
201 were then mixed with SSIFs with different compositions: bile extract (with and without $CaCl_2$); pure
202 bile salts (with and without $CaCl_2$); and, pure bile salts with $CaCl_2$ and phospholipids (DOPC). The
203 influence of SSIF composition and carrier oil type on particle characteristics after digestion were
204 then measured.

205 3.1.1. Influence on particle size

206 For both MCT-VE and LCT-emulsions, the initial systems (before digestion) had monomodal
207 particle size distributions and relatively small mean particle diameters (d = 140-150 nm) (**Figures**
208 **2a-c**). The properties of the vitamin-fortified emulsions were also measured after they were
209 exposed to the simulated small intestinal model: mean particle diameters (**Figure 2a**) and particle
210 size distributions (**Figures 2b and 2c**).

211 After passage through the simulated small intestinal stages, the mean particle diameters of all
212 samples increased and there was evidence of large particles in the particle size distributions (**Figures**
213 **2a-c**). The composition of the simulated small intestinal fluids (SSIFs) had a pronounced influence
214 on the particle size of the emulsions after digestion. The presence of bile extract in the SSIF caused
215 little change in particle size, but the presence of pure bile salts (NaC and NaDC) caused an
216 appreciable increase in particle size. The presence of calcium ions in the SSIFs caused a large
217 increase in mean particle diameter (**Figure 2a**) and there was evidence of large particles in the
218 particle size distributions for both MCT- and LCT-emulsions (**Figures 2b and 2c**). The presence of
219 these large particles indicates a marked change in the structure of the systems after exposure to small
220 intestinal conditions, which may be due to several physicochemical phenomena. The pancreatic
221 lipase in the SSIFs will adsorb to the lipid droplet surfaces and convert the triacylglycerols (TAGs)
222 into free fatty acids (FFAs) and monoacylglycerols (MAGs). The products of lipid hydrolysis may

223 move into the surrounding aqueous phase or remain at the droplet surfaces depending on their
224 water-dispersibility, which is related to their chain length [19, 22]. Long chain FFAs tend to remain at
225 the droplet surface (in the absence of bile salts or calcium), whereas short and medium chain FFAs
226 tend to move into the aqueous phase. Lipid digestion may therefore reduce the size of the initial
227 lipid droplets due to removal of FFA and MAG digestion products from their surfaces [14, 15]. On the
228 other hand, partially digested lipid droplets may be more prone to droplet coalescence due to the
229 change in their interfacial properties, which would lead to an increase in particle size [14, 15]. It should
230 also be noted that the light scattering instrument is sensitive to all kinds of particles that scatter light
231 within the sample, which includes any mixed micelles or insoluble calcium complexes formed after
232 digestion [40].

233 The measured particle size did not change much after vitamin-fortified MCT- or LCT-emulsions
234 were exposed to SSIFs in the absence of calcium, regardless of whether bile extract or pure bile salts
235 were used (**Figures 2a-c**). This may have occurred because some of the lipid droplets were not
236 digested and retained their original size, but this is unlikely since the pH-stat measurements
237 (described below) indicated that lipid digestion had occurred. It is therefore possible that the mixed
238 micelles formed by lipid digestion were of a similar size to the original lipid droplets in the system.

239 In general, the droplet sizes were appreciably larger when the SSIFs contained bile salts than
240 when they contain bile extract (**Figure 2a**). Bile extract from porcine is a complex mixture that
241 contains various bile salts, phospholipids and other components, whereas the bile salts only
242 contained pure NaC and NaDC. Hence, the concentration of actual bile salts in the system would
243 be higher for the pure bile salts than for the bile extract, which may have led to the formation of more
244 insoluble complexes with calcium. In addition, the mixed micelles formed by pure bile salts may
245 have been larger than those formed by bile extract, *e.g.,* pure bile salts may have formed large vesicle
246 structures, whereas some of the components in bile extract may have promoted disruption of these
247 structures. Nevertheless, further work is required using microscopy methods to identify the precise
248 nature of the mixed micelles formed by different sources of bile salts.

249 Another complication associated with interpreting the results of light scattering measurements in
250 complex colloidal dispersions is associated with data analysis. The software used to calculate the
251 particle size distribution of a colloidal dispersion from its light scattering pattern usually assumes
252 that the particles are spherical, dilute, and have well-defined refractive indices. However, the

253 colloidal dispersions resulting from lipid digestion contain a complex mixture of particles with
254 different compositions and structures, such as undigested lipid droplets, partially digested lipid
255 droplets, micelles, vesicles, and various other colloidal structures. Light scattering results should
256 therefore be treated with some caution for this type of complex colloidal dispersion.

257 **3.1.2 Influence on particle charge characteristics**

258 In this section, changes in the electrical charge on the particles in the samples after digestion
259 were measured to provide some information about possible changes in interfacial composition
260 (**Figure 3**). Initially, all of the oil droplets coated by bile salts were highly negatively charged
261 (-61.6 mV for MCT emulsions and -63.7 mV for LCT emulsions), which can be attributed to
262 ionization of the bile salts. At neutral pH, bile salts have an appreciable negative charge due to the
263 presence of anionic carboxyl groups, *i.e.,* $-COO^-$ [46]. The particles in all the samples were negative
264 after exposure to the simulated small intestine stage, although there was some reduction in the
265 magnitude of their negative charge (**Figure 3**). The negative charge on the particles may be a result
266 of some of the initial bile salts remaining at the droplet surfaces, as well as due to the absorption of
267 other anionic surface active species from the digestion medium. The reduction in droplet charge
268 may have been because some of the bile salts were displaced by FFAs or because of the increase in
269 ionic strength in the system [47].

270 The charge on the particles present in the digesta was less negative in the presence of calcium
271 ions (-21 to -32 mV) than in their absence (-44 to -52 mV) for both carrier oil types, which can be
272 attributed to binding of cationic Ca^{2+} ions to the surfaces of the anionic droplets and mixed micelles,
273 as well as some electrostatic screening effects [47]. The negative charge was higher on the particles
274 present in LCT-emulsions than those present in MCT-emulsions after digestion (**Figure 3**) which
275 may be due to the fact that glyceryl trioleate contains long chain fatty acids (C18:1) that accumulate
276 at oil-water interfaces, whereas glyceryl trioctanoate contains medium chain fatty acids (C8:0) that
277 tend to move into the surrounding aqueous phase [48].

278 It should be noted that it is not clear exactly what types of particles are detected by an
279 electrophoresis instrument in a complex colloidal dispersion that contains different types of charged
280 particles that scatter light. Moreover, since measurements were made on diluted and stirred samples,
281 the nature of the particles in the measurement cells might be different from the particles in the

282 original undiluted samples. One should therefore be cautious when interpreting the results from
283 electrophoresis measurements on this type of complex colloidal dispersion.

284 3.2 Impact of SSIF composition and carrier oil type on lipid digestion

285 In this part of the study, the influence of carrier oil type and SSIF composition on the rate and
286 extent of lipid digestion was measured using the pH-stat method, which is widely used in
287 pharmaceutical and food research for this purpose [32, 35, 49-53]. The principle of the pH-stat method is
288 to measure the volume of alkaline solution (0.25 M NaOH) required to neutralize the free fatty acids
289 (FFAs) released from a sample when incubated in SSIFs containing lipase. This information is then
290 used to calculate the percentage of FFAs released from the sample, assuming that a maximum of two
291 FFAs are released per triglyceride molecule.

292 Generally, there was a rapid initial increase in the volume of NaOH added to the emulsions
293 during the first few minutes of incubation in SSIFs, followed by a more gradual increase at longer
294 times (**Figure 4**). This result suggests that lipases in the SSIFs were able to quickly adsorb to the
295 lipid droplet surfaces and convert encapsulated TAGs into FFAs and MAGs. Nevertheless,
296 vitamin-fortified emulsions prepared using different types of carrier oil exhibited quite different
297 behavior. In the absence of calcium, the rate and extent of lipid digestion was appreciably higher
298 for MCT-emulsions than for LCT-emulsions (**Figure 4e**), which is in good agreement with previous
299 studies [19, 22, 31]. As mentioned earlier, long-chain FFAs accumulate at lipid droplet surfaces in the
300 absence of calcium ions [48], which inhibits digestion by preventing lipase molecules from reaching
301 non-digested TAGs at the core of the lipid droplets [34].

302 The influence of calcium and phospholipid addition on the titration of FFAs during *in vitro*
303 digestion was also studied (**Figure 4**). For MCT-emulsions, the presence of calcium ions in the
304 SSIF did not cause an appreciable alteration in lipid digestion (**Figures 4a and 4b**). However, for
305 LCT-emulsions, the addition of calcium ions led to an appreciable increase in the final amount of
306 lipid digestion products generated (**Figures 4c and 4d**). These measurements clearly show that
307 calcium ions have a major impact on lipid digestion in LCT-emulsions but not in MCT-emulsions,
308 which is in agreement with previous research [40]. This result may be explained by a number of
309 physicochemical mechanisms. First, a certain amount of calcium is required as a co-factor to
310 activate pancreatic lipase [54, 55]. Thus, the lower extent of FFA production in the absence of calcium

311 may be partly due to the fact that the enzyme was not in its most active form. However, this is
312 unlikely to be important because lipid digestion still occurred in the MCT-emulsions in the absence
313 of calcium. Second, calcium ions bind to long-chain FFAs generated during the digestion of
314 emulsified LCT and form insoluble calcium soaps that remove them from the droplet surfaces [35, 36, 39,
315 40]. The precipitation of these long-chain fatty acids enables lipase molecules to come into close
316 contact with the remaining non-digested lipids and facilitate their digestion [56]. The digestion of
317 emulsified MCT is less dependent on calcium ions because the lipid digestion products
318 (medium-chain FFAs) are more water-dispersible and rapidly move into the surrounding aqueous
319 phase, thereby enabling lipase to continue operating at the droplet surfaces. Calcium ions may also
320 impact lipid digestion by affecting other characteristics of emulsions, such as droplet aggregation [57,
321 58]. Anionic lipid droplets may become flocculated in the presence of cationic calcium ions due to
322 ion binding and electrostatic scattering screening effects, which may reduce the ability of lipase to
323 interact with the lipid droplet surfaces [59, 60].
324 For both MCT-VE and LCT-emulsions, the addition of phospholipids (DOPC) into the SSIFs
325 increased the final extent of lipid digestion. This may have occurred because phospholipids
326 facilitated the ability of the lipase to interact with the emulsified TAGs, or because the phospholipids
327 were themselves hydrolyzed and released FFAs. Based on the amount of DOPC (36 mg) present in
328 the SSIF, and the assumption that one FFA is released per phospholipid molecule, we calculated that
329 about 0.18 mL of 0.25 M NaOH would be required to neutralize any fatty acids produced due to
330 phospholipid hydrolysis. This value is quite close to the difference between the volumes of NaOH
331 required to neutralize calcium-containing samples in the presence and absence of DOPC (**Figures 4b**
332 **and 4d**). We therefore conclude that the increased amount of alkali required for the samples
333 containing DOPC is mainly due to the hydrolysis of the phospholipid by digestive enzymes in the
334 SSIFs.

335 **3.3 Impact of SSIF composition and carrier oil type on vitamin E bioaccessibility**

336 The impact of SSIF composition and carrier oil type on the bioaccessibility of vitamin E after
337 passage through the simulated small intestine was also examined. The bioaccessibility was
338 determined by incubating the emulsions in SSIFs for 2 hours, centrifuging the resulting digesta, and
339 then determining the concentration of vitamin E in the micelle phase and overall digesta using

340 HPLC.

341 The overall appearance of the digesta after exposure to SSIFs depended on calcium content
342 (**Figure 5**). In the absence of calcium, samples separated into a thick white layer at the top
343 ("cream"), a clear or slightly turbid layer in the middle ("micelle phase"), and a thin white layer at
344 the bottom ("sediment"). The white layer at the top probably consisted of non-digested fat droplets
345 and possibly some large mixed micelle structures (which are less dense than water), while the white
346 layer at the bottom probably contained insoluble matter such as bile salt or protein complexes (which
347 are denser than water). In the presence of calcium, we only observed a single white layer
348 ("sediment") at the bottom of the samples with a clear or slightly turbid layer above ("micelle
349 phase"). The fact that a cream layer was not observed in the samples containing calcium can be
350 attributed to two factors. First, calcium promoted digestion of the lipid phase (**Figure 5**), and so
351 there would be less non-digested lipid droplets present. Second, cationic calcium ions (Ca^{2+})
352 formed dense insoluble aggregates with anionic species, such as bile salts and free fatty acids, which
353 sedimented to the bottom of the tubes.

354 The mixed micelles formed in the human body are compositionally and structurally complex
355 colloidal dispersions whose properties depend on the nature of any co-ingested lipids [18, 19]. Mixed
356 micelles contain bile salts, phospholipids, and cholesterol from the small intestinal fluids, as well as
357 monoacylglycerols (MAG) and free fatty acids (FFAs) from any lipid digestion products. These
358 surface-active lipids self-assemble into the mixed micelle phase, which may contain micelles,
359 vesicles, and liquid crystalline phases that vary in composition, dimensions, and structure [61, 62]. The
360 micelle phase was therefore passed through a 450 nm pore size filter before measuring the
361 bioaccessibility to more closely simulate gastrointestinal conditions [63, 64]. Lipophilic bioactive
362 components solubilized within mixed micelles must pass through the mucus layer before they can be
363 absorbed by the human body. The mucus layer acts as a biological filter that only allows particles
364 smaller than about 400 nm to pass through [63]. Filtering the micelle layer prior to analysis may
365 therefore provide a more accurate representation of the potential bioavailability of a lipophilic
366 compound that needs to be transported by mixed micelles through the mucus layer.

367 In the presence of calcium ions, the size of the particles in the micelle phases collected from the
368 LCT-emulsions were slightly larger than those collected from the MCT-emulsions (**Figure 6**), which
369 suggests that that the colloidal structures in the micelle phase formed by LCT digestion products

370 were larger than these formed by MCT digestion products. One possible explanation for this
371 observation is that long chain fatty acids form more vesicles or liquid crystals (which are larger than
372 simple micelles) than medium chain fatty acids, but microscopy analysis of the mixed micelle phases
373 would be required to demonstrate this.

374 The nature of the bile salts present in the SSIFs also had an influence on the size of the
375 structures formed in the mixed micelle phase. The particle size was larger when the SSIF contained
376 bile extract, than when it contained pure bile salts (**Figure 6**). Bile extract contains a mixture of
377 bile salts, phospholipids, and other components [40, 65], which may have led to the formation of larger
378 mixed micelles. Indeed, when phospholipids (DOPC) were added to the SSIF containing bile salts
379 and calcium ions there was an appreciable increase in the size of the colloidal structures present in
380 the micelle phase (**Figure 6**). In addition, bile extract may have contained some insoluble matter
381 that also contributed to the light scattering signal.

382 The bioaccessibility of the vitamin E was determined by measuring the concentrations of
383 α-tocopherol acetate and α-tocopherol in the micelle phase and within the total digesta after *in vitro*
384 digestion (**Figure 7**). Calcium ions had a major impact on the bioaccessibility of vitamin E, which
385 depended strongly on the nature of the carrier oil used. For the MCT-emulsions, the addition of
386 calcium ions to the SSIF led to an appreciable decrease in the bioaccessibility of vitamin E,
387 regardless of the nature of bile salts used. For example, the vitamin bioaccessibility decreased from
388 around 24% to 9% when calcium was added to SSIFs containing bile extract, whereas it decreased
389 from around 31% to 1% when calcium was to SSIFs containing pure bile salts. It is possible that
390 cationic calcium ions formed insoluble precipitates with mixed micelles containing solubilized
391 vitamin E, thereby reducing the amount of vitamin E present within the micelle phase. Conversely,
392 for the LCT-emulsions, the addition of calcium ions to the SSIF led to an appreciable increase in the
393 bioaccessibility of vitamin E. For example, the vitamin bioaccessibility increased from around 22%
394 to 32% when calcium was added to SSIFs containing bile extract, whereas it increased from around
395 11% to 37% when calcium was to SSIFs containing pure bile salts. In addition, there was a further
396 increase (to around 66%) when phospholipids (DOPC) were incorporated into the SSIFs for the
397 LCT-emulsions. Previous researchers have also reported that phospholipids can increase the
398 bioavailability of lipophilic bioactive components [66-69]. The solubilization of lipophilic
399 components in the micelle phase usually depends on the total amount of mixed micelles available for

400 transporting them across the mucus layer. Our pH-stat experiments show that a higher amount of
401 lipid digestion products (FFAs and MAGs) are formed when calcium is present during the digestion
402 of LCT-emulsions, and so there should be a greater amount of mixed micelles present to solubilize
403 the vitamin E. In addition, a greater amount of vitamin E should have been released from the lipid
404 droplets when more TAGs were digested. The addition of calcium ions to the LCT-emulsions may
405 therefore increase the amount of vitamin E in the mixed micelle phase. Nevertheless, one might
406 still expect the calcium ions to cause some precipitation of the mixed micelles (as with the
407 MCT-emulsions), which would reduce the amount of vitamin E in the micelle phase. Our results
408 suggest that the greater release of vitamin E from the lipid droplets and the higher amount of mixed
409 micelles formed in the presence of calcium, outweigh the precipitation effect for the LCT-emulsions.
410 These results may have important implications for the design of effective emulsion-based delivery
411 systems for vitamin E. Calcium is normally present within the fluids secreted by the human
412 gastrointestinal tract. This calcium may reduce the bioavailability of vitamin E delivered in
413 MCT-emulsions, which might be overcome by incorporating calcium chelating agents in the delivery
414 system (such as EDTA or alginate). On the other hand, calcium may increase the bioavailability of
415 vitamin E in LCT-emulsions, and therefore it may be advantageous to avoid the presence of calcium
416 chelating agents in these delivery systems or to supplement them with additional calcium.

417 Under realistic digestion conditions (*i.e.,* samples containing bile salts, calcium and
418 phospholipids), the bioaccessibility of vitamin E was appreciably higher when it was encapsulated in
419 LCT-emulsions than in MCT-emulsions (**Figure 7**). The long-chain free fatty acids ($C_{18:1}$) arising
420 from the lipolysis of glycerol trioleate (LCT) can presumably form colloid structures with a larger
421 solubilization capacity than the medium chain fatty acids ($C_{8:0}$) generated from the hydrolysis of
422 glycerol trioctanoate (MCT). The α-tocopherol molecule has a non-polar chain with 14 carbon
423 atoms (C_{14}), which is presumably too long to be accommodated into the micelles or vesicles formed
424 by medium-chain fatty acids, but short enough to be incorporated into those formed by long chain
425 fatty acids. Researchers in the pharmaceutical area have also reported that the bioaccessibility of
426 some highly oil-soluble drugs is greater when LCT was used as a carrier oil rather than MCT [70, 71].
427 It should be noted that it is the dimensions of the lipophilic structures within the mixed micelle phase
428 that can incorporate α-tocopherol molecules, such as the hydrophobic core of simple micelles or the
429 bilayers of vesicles, rather than their overall dimensions that is important. For example, a vesicle

430 can have relatively large overall dimensions (> 100 nm), but it can only accommodate a lipophilic
431 molecule if it can fit within the bilayers formed by the fatty acid tails (a few nm).

432 ### 3.4. Impact of SSIF composition and carrier oil type on vitamin E conversion

433 The esterified form of α-tocopherol is often used in foods and other commercial products rather
434 than the free form because it is more stable to oxidation during processing, transportation and storage
435 [72, 73]. Previous research has shown that the molecular form of vitamin E has a major impact on the
436 bioaccessibility of vitamin E, *i.e.,* α-tocopherol *versus* α-tocopherol acetate [27-30]. Thus, we
437 determined the amount of α-tocopherol acetate converted to α-tocopherol after *in vitro* digestion
438 (**Figure 8**). Our previous research using food-grade oils showed that carrier oil type had an
439 appreciable impact on the hydrolysis of α-tocopherol acetate to α-tocopherol, with the extent of
440 conversion being about 29% for LCT-emulsions and 17% for MCT-emulsions [24]. This result
441 suggests that the conversion of α-tocopherol acetate to α-tocopherol occurred more readily when
442 LCT was used as the carrier oil than when MCT was used [24]. In the current study, we used purified
443 LCT and MCT carrier oils to provide further insights into this important effect.

444 For MCT-emulsions, the SSIF composition did not have an appreciable impact on the
445 conversion of α-tocopherol acetate to α-tocopherol (**Figure 8**). Conversely, for LCT-emulsions, the
446 SSIF composition had a major impact on α-tocopherol acetate hydrolysis. For the LCT-emulsions,
447 the conversion of α-tocopherol acetate to α-tocopherol increased when calcium and phospholipid
448 were incorporated into the SSIFs (**Figure 8**). There therefore appeared to be a correlation between
449 the bioaccessibility of vitamin E and the hydrolysis of α-tocopherol acetate. It is likely that
450 α-tocopherol acetate can only be hydrolyzed by digestive enzymes after it is released from the
451 interior of the fat droplets [74]. Hydrolysis may occur at the lipid droplet surfaces or after the
452 α-tocopherol acetate is incorporated into mixed micelles, which would account for the increase in
453 hydrolysis with increasing bioaccessibility. This effect may also account for that fact that the extent
454 of hydrolysis was greater for the LCT-emulsions than the MCT-emulsions in the presence of calcium
455 ions (**Figure 8**).
456

457 ## 4. Conclusions

458 The purpose of this study was to identify the key factors impacting the bioaccessibility of

459 emulsified α-tocopherol acetate using a simulated small intestine model. We have shown that the
460 rate and extent of lipid digestion was higher for MCT-emulsions than for LCT-emulsions, which was
461 attributed to differences in the water-dispersibility of the medium and long chain fatty acids formed
462 during lipolysis. The addition of calcium ions to the SSIFs greatly increased the extent of lipid
463 digestion for LCT-emulsions, but had little effect on MCT-emulsions, which was attributed to the
464 ability of calcium ions to remove long-chain fatty acids from droplet surfaces. The addition of
465 calcium ions and phospholipids into the SSIFS also had a major impact on the bioaccessibility of
466 vitamin E depending on carrier oil type. The addition of calcium ions greatly improved the
467 bioaccessibility of vitamin E in LCT-emulsions, but reduced it in MCT-emulsions. Finally, calcium
468 addition increased the conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion of
469 LCT-emulsions, but had little effect on α-tocopherol acetate hydrolysis in MCT-emulsions. A
470 schematic representation of the important physicochemical events occurring with the gastrointestinal
471 tract based on our results is shown in Figure 9.

472 In summary, our results suggest that the bioaccessibility of vitamin E encapsulated in
473 emulsion-based delivery systems is strongly influence by carrier oil type, bile salt type, calcium ions,
474 and phospholipids. This information is important for developing effective emulsion-based delivery
475 systems for oil-soluble vitamins and testing their potential efficacy.

476 5. Acknowledgements

477 The authors thank BASF for providing the vitamin E acetate used in these experiments. This
478 material is based upon work supported by the Cooperative State Research, Extension, Education
479 Service, United State Department of Agriculture, Massachusetts Agricultural Experiment Station and
480 United States Department of Agriculture, CREES, NRI, AFRI (2011-03539, 2013-03795,
481 2011-67021, and 2014-67021**)** Grants.

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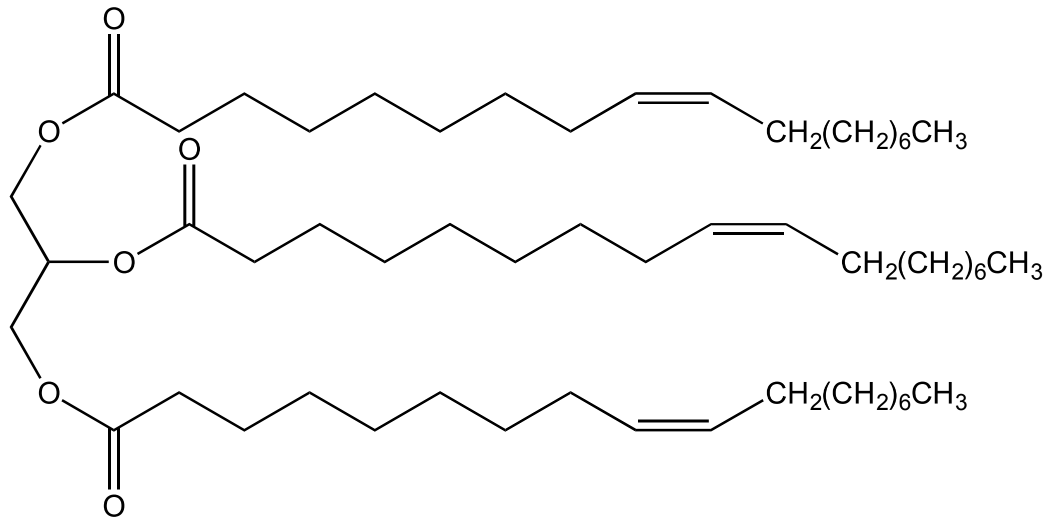
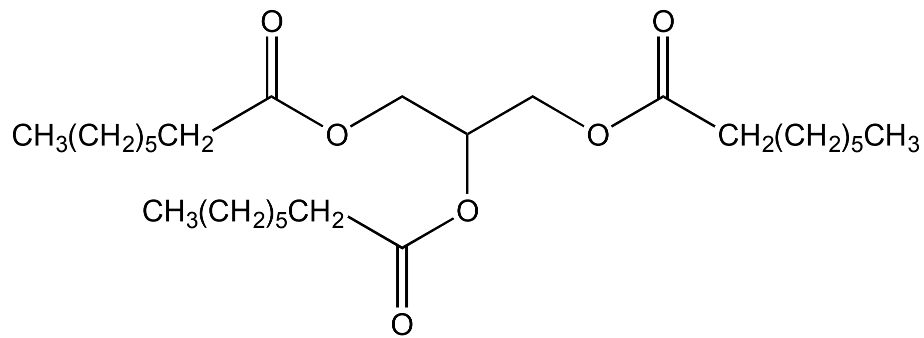
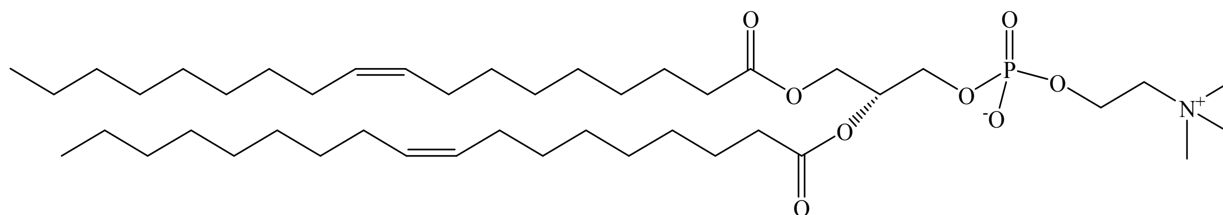
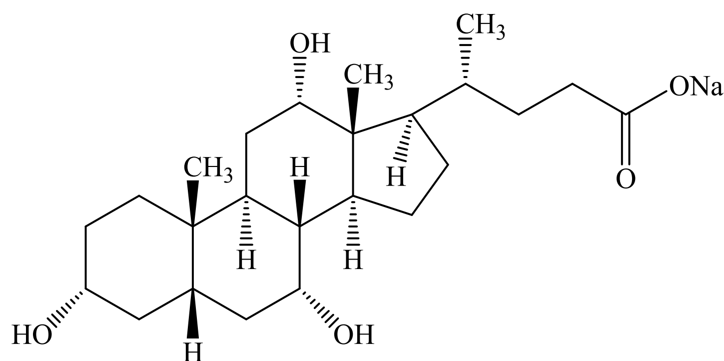


Glyceryl trioleate (C18:1)



Glyceryl trioctanoate (C8:0)





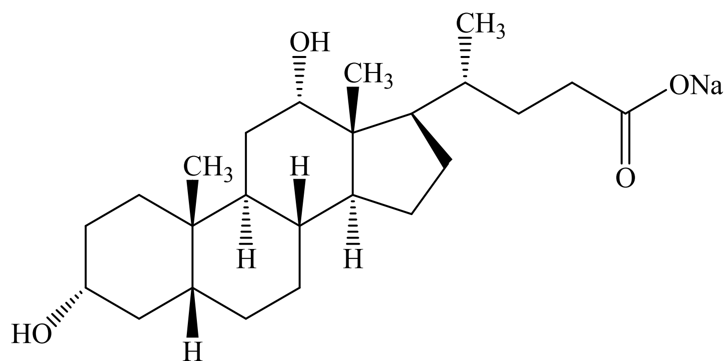


Figure 1. Structures of the different components used in this study: (i) *digestible lipids*: long chain triglyceride (glyceryl trioleate, C18:1); medium chain triglyceride (glyceryl trioctanoate, C8:0); (ii) simulated small intestine fluid (SSIF) components: sodium cholate (NaC); sodium deoxycholate (NaDC), and phospholipid (DOPC).

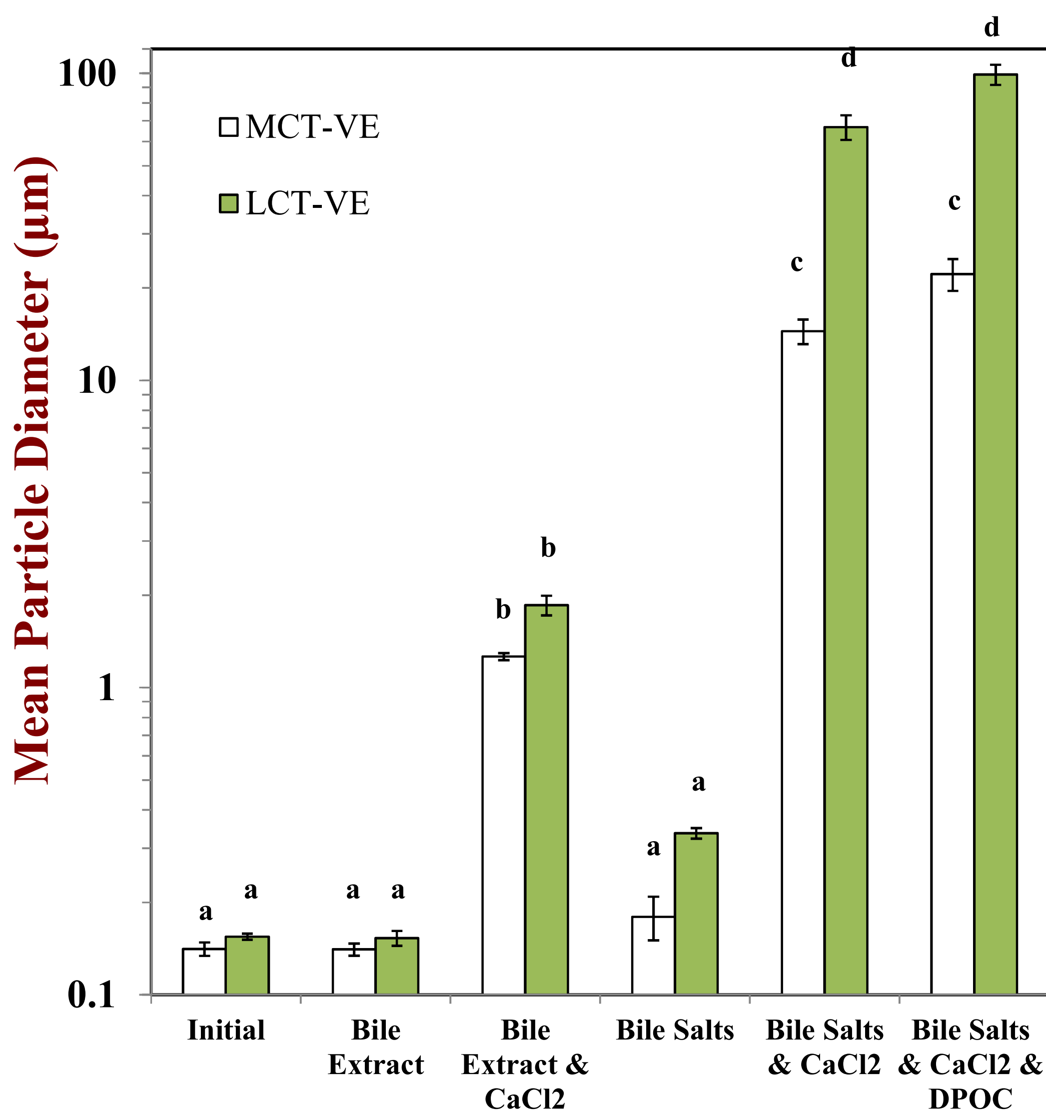


Figure 2a: Influence of carrier oil type and simulated small intestine fluid composition on the mean particle diameter (d_{32}) of oil-in-water emulsions after passing through a simulated small intestine tract. Data are means±SD for n = 4 independent simulated digestions. The presence of a different letter above error bars indicates that particle sizes after *in vitro* digestion were significantly ($p < 0.05$) affected by the addition of calcium and carrier oil type.

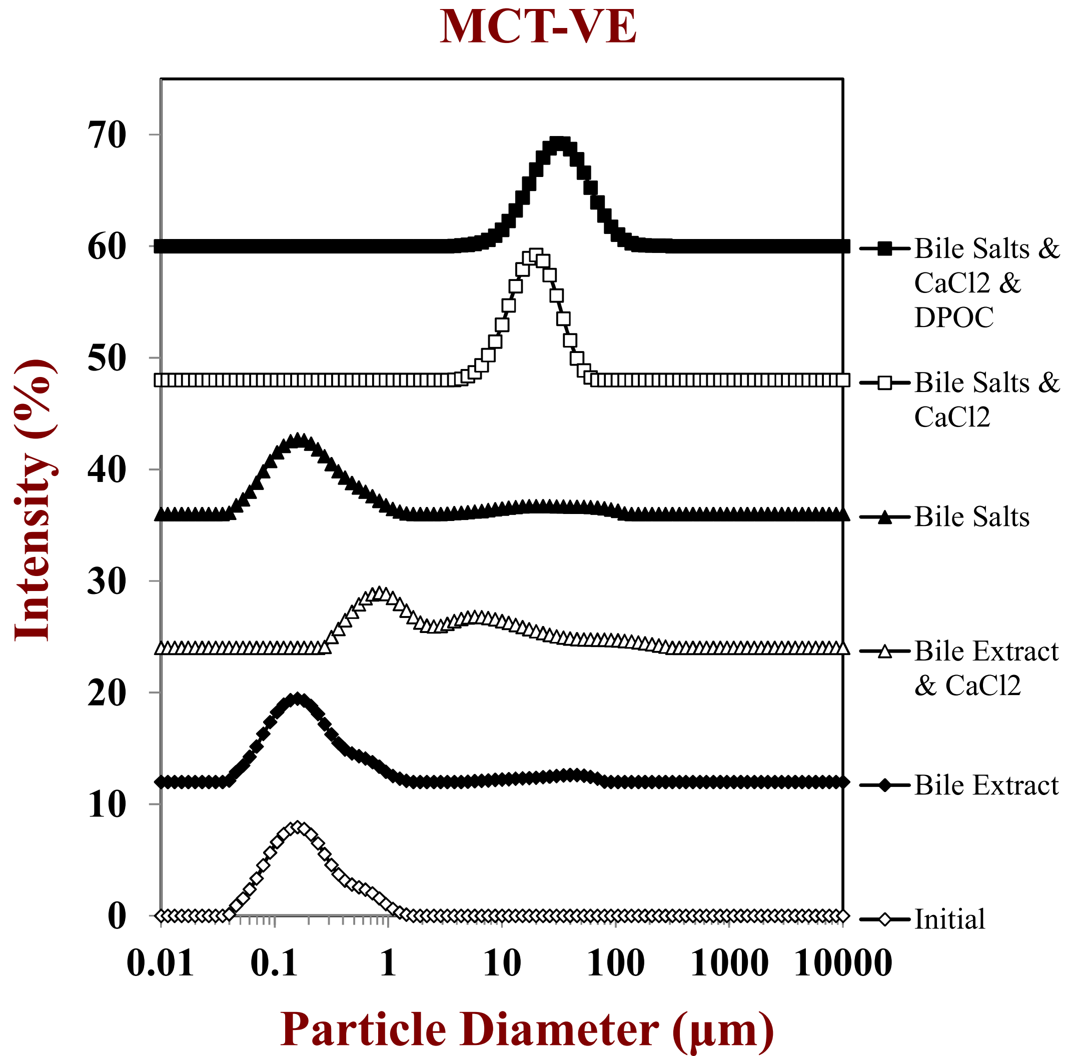


Figure 2b Influence of carrier oil type and simulated small intestine fluid composition on the particle size distribution of MCT-VE emulsions passing through the simulated small intestine tract.

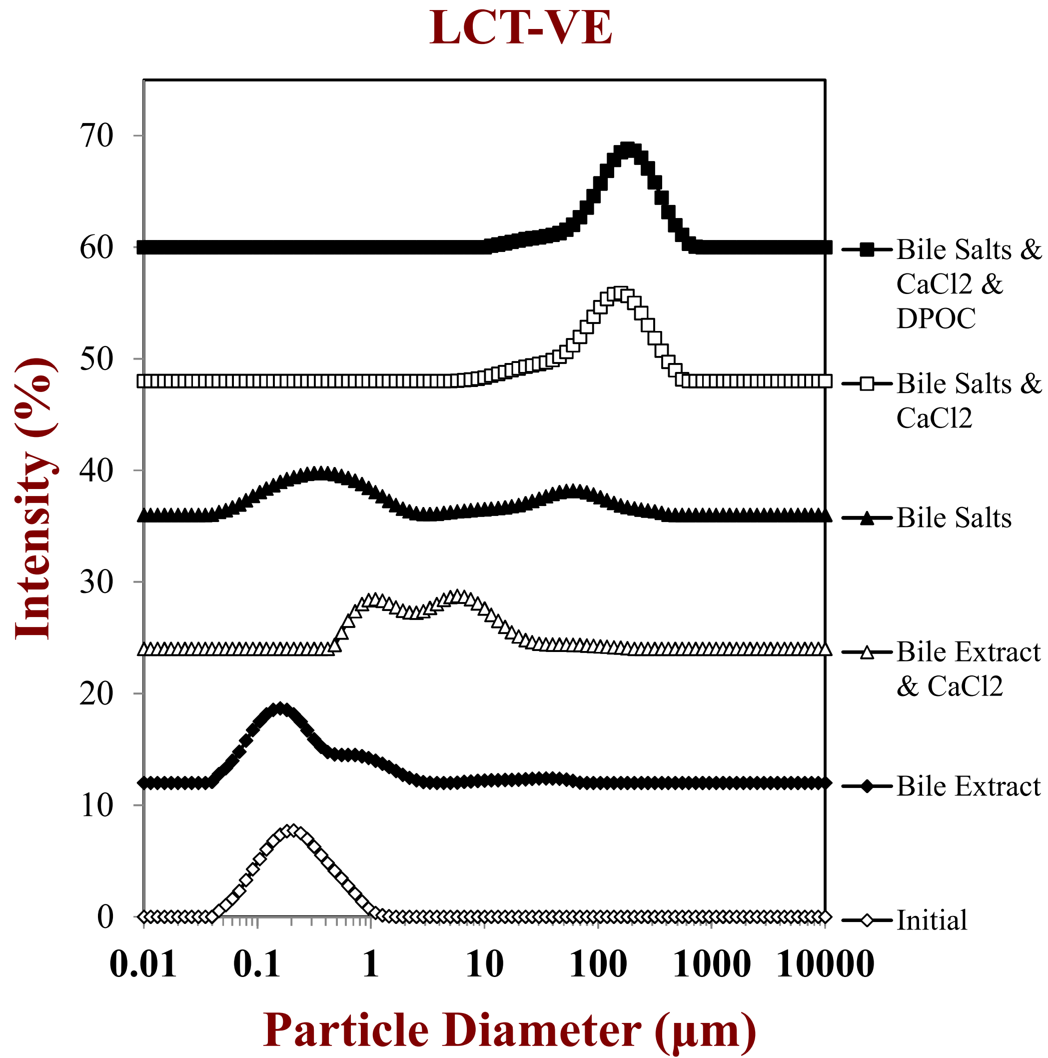


Figure 2c Influence of carrier oil type and simulated small intestine fluid composition on the particle size distribution of LCT-VE emulsions passing through the simulated small intestine tract.

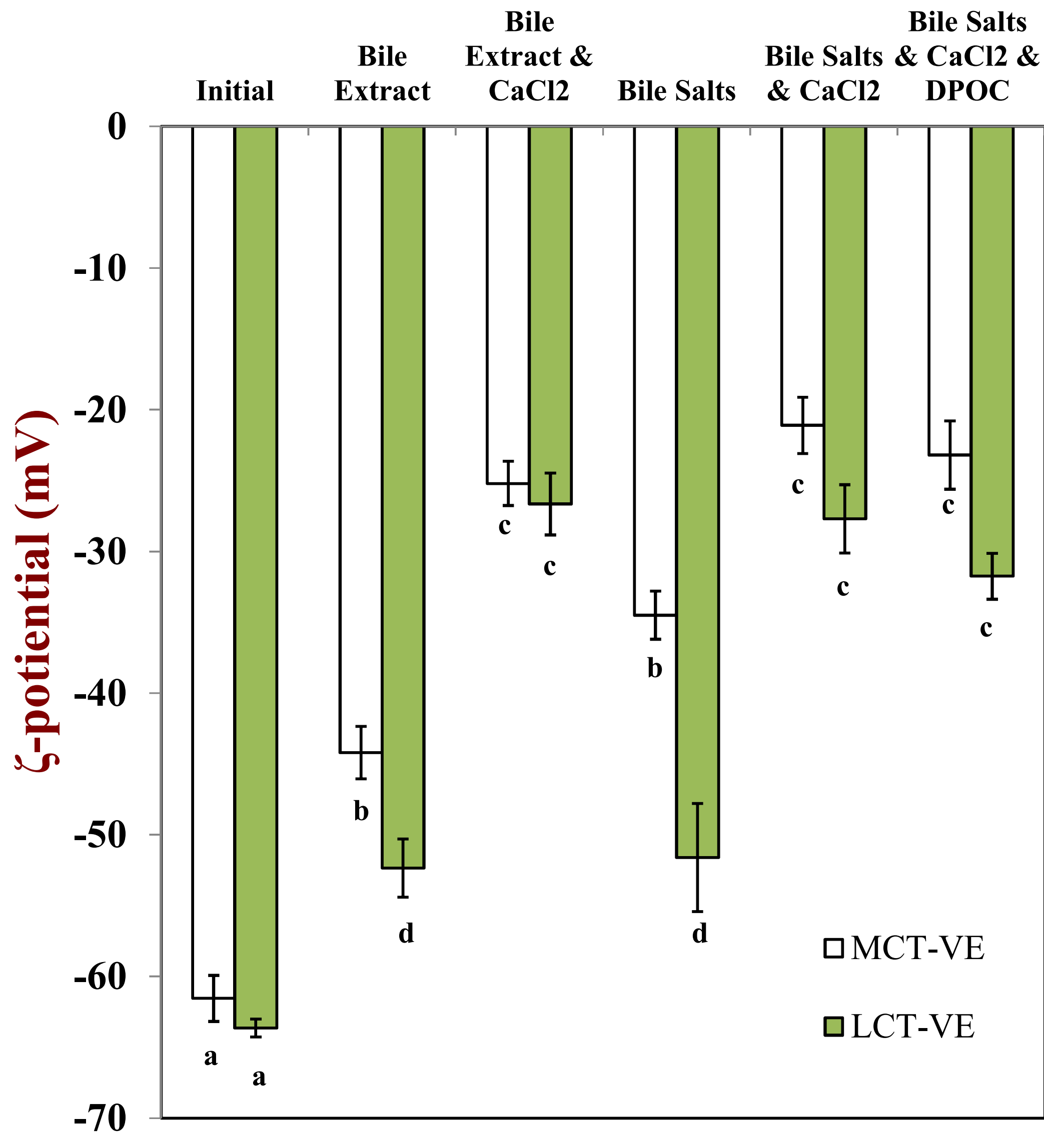


Figure 3. Influence of carrier oil type and SSIF composition on the electrical characteristics (ζ-potential) of the particles in oil-in-water emulsions passed through simulated small intestine. Data are means±SD for n = 4 independent simulated digestions. Letters show samples with significant differences ($p < 0.01$)

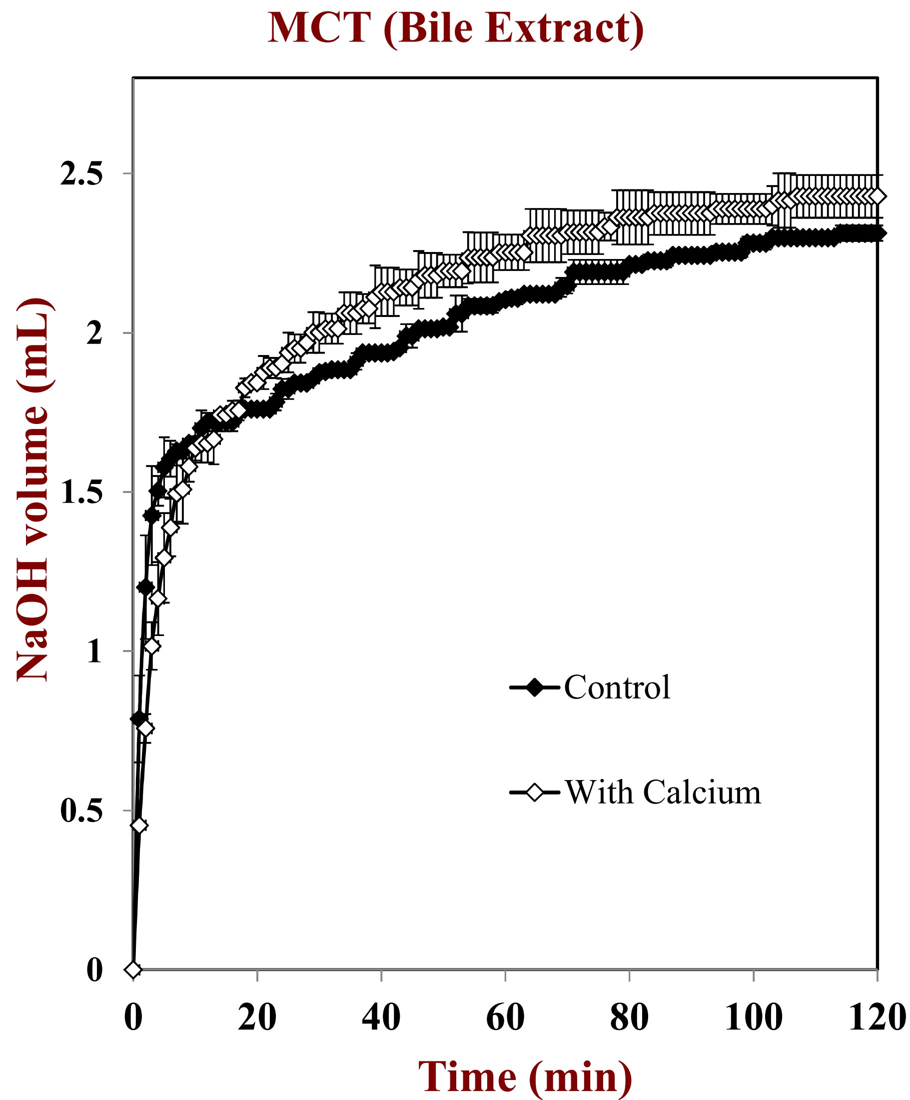


Figure 4a Effect of calcium ($CaCl_2$) on the rate and extent of lipid digestion in MCT-VE emulsions determined using a pH-stat method with SSIF containing bile extract. Data are means ± SD for n = 4 independent simulated digestions.

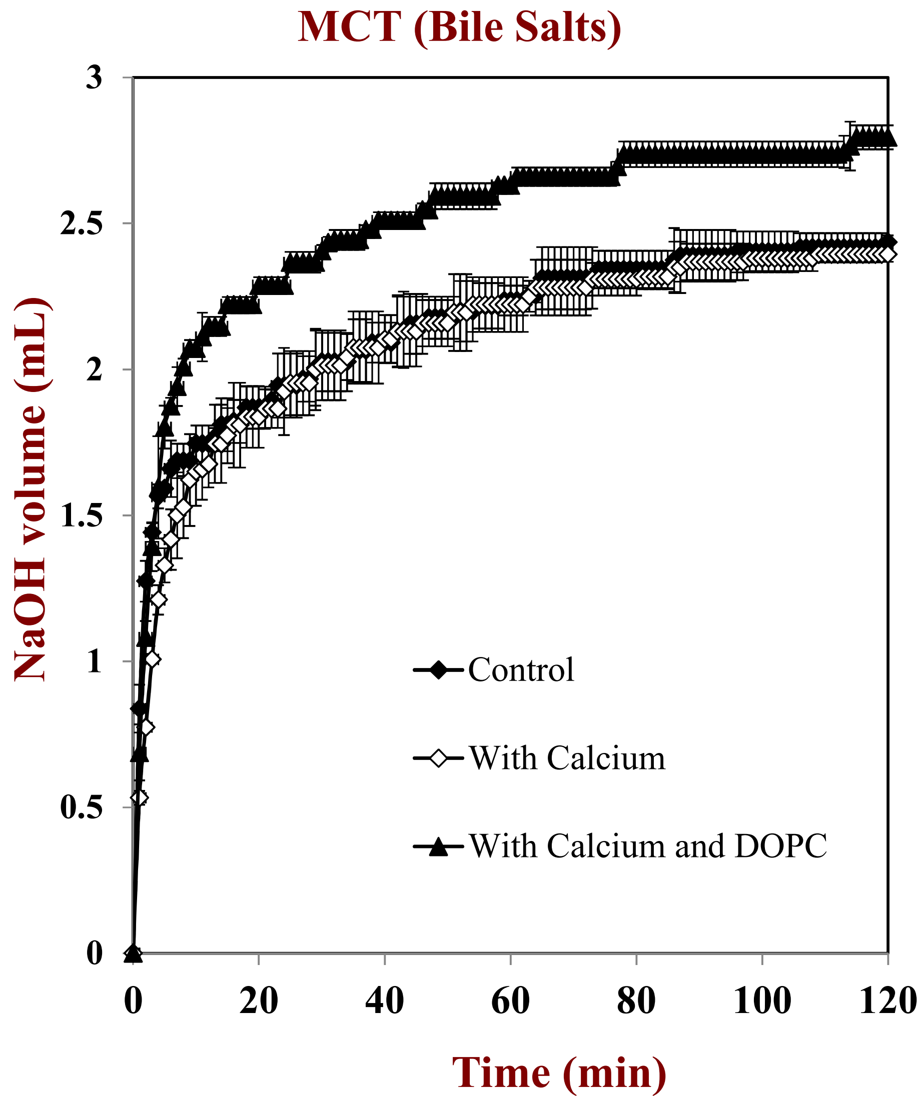


Figure 4b Effect of calcium ($CaCl_2$) and phospholipids (DOPC) on the rate and extent of lipid digestion in MCT-VE emulsions determined using a pH-stat method with SSIF containing pure bile salts (NaC and NaDC). Data are means ± SD for n = 4 independent simulated digestions.

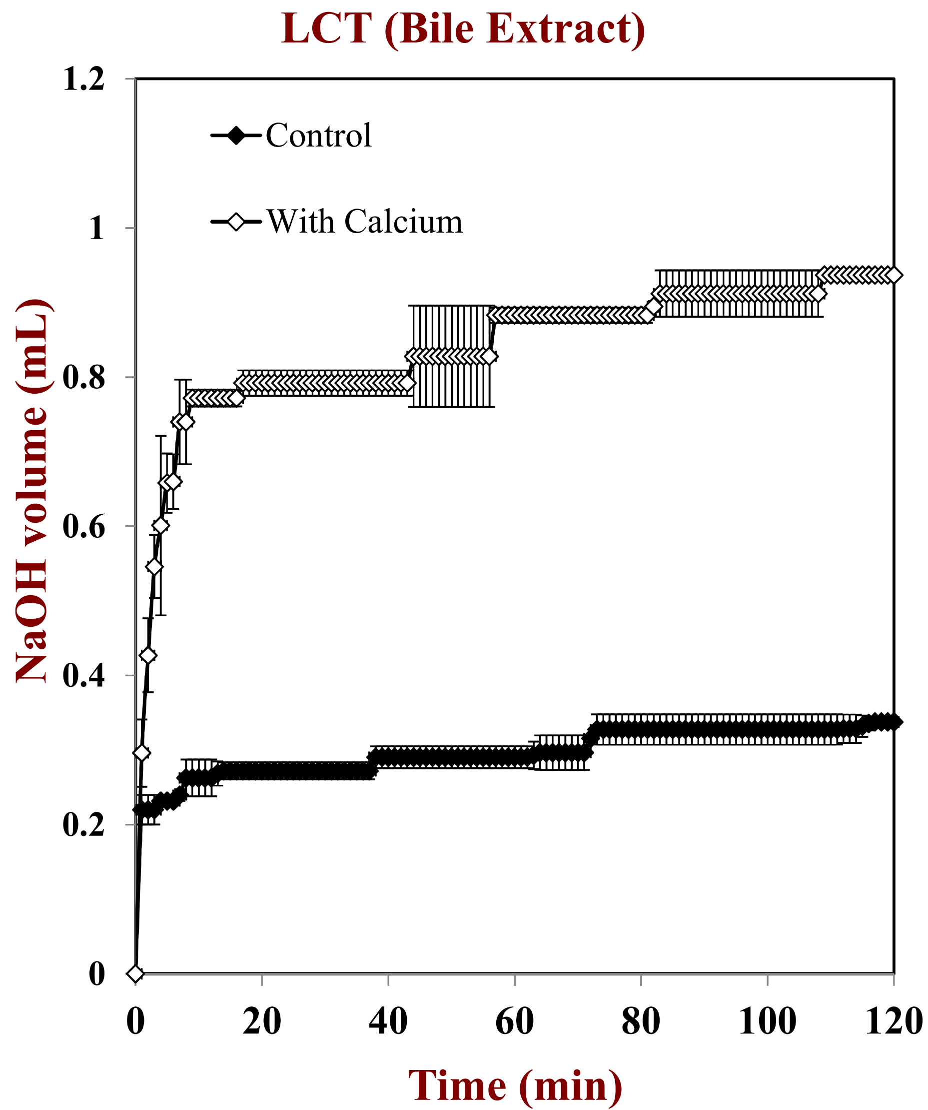


Figure 4c Effect of calcium ($CaCl_2$) on the rate and extent of lipid digestion in LCT-VE emulsions determined using a pH-stat method with SSIF containing bile extract. Data are means ± SD for n = 4 independent simulated digestions.

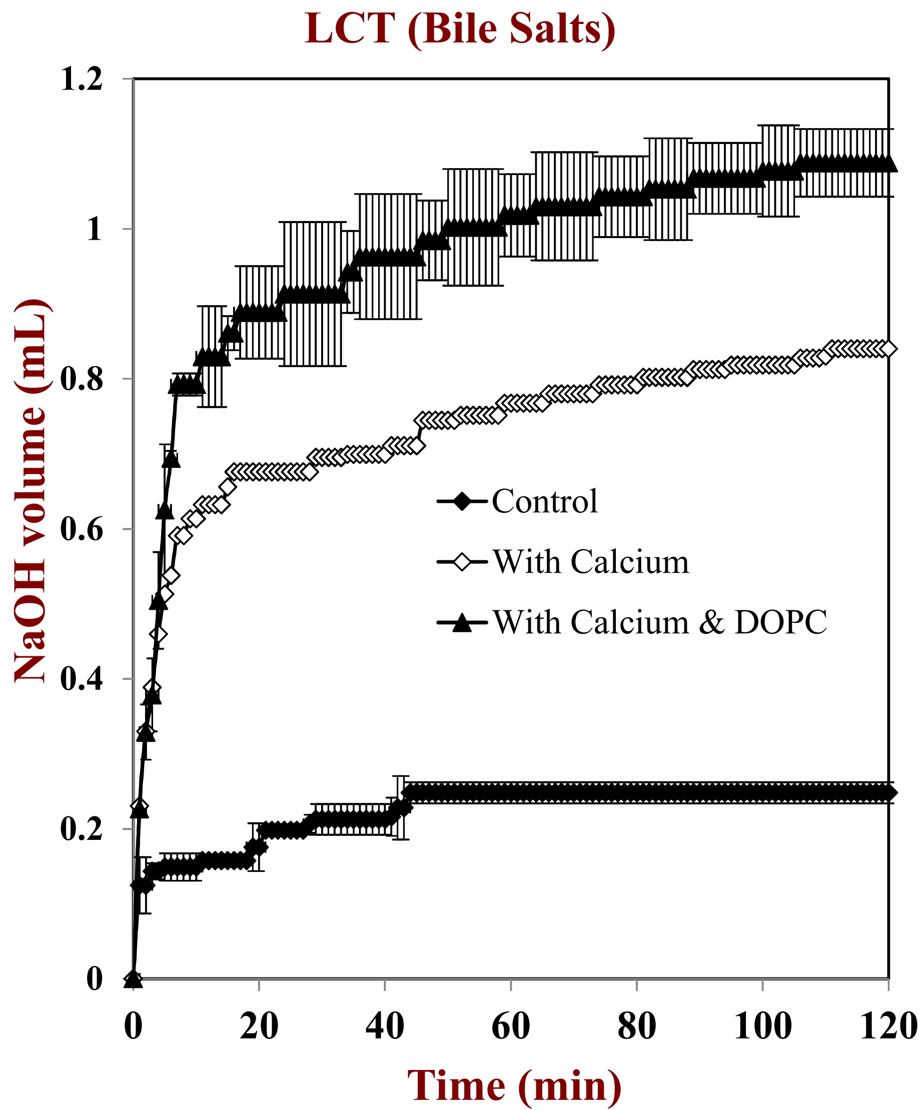


Figure 4d Effect of calcium ($CaCl_2$) and phospholipids (DOPC) on the rate and extent of lipid digestion in LCT-VE emulsions determined using a pH-stat method with SSIF containing pure bile salts (NaC and NaDC). Data are means ± SD for n = 4 independent simulated digestions.

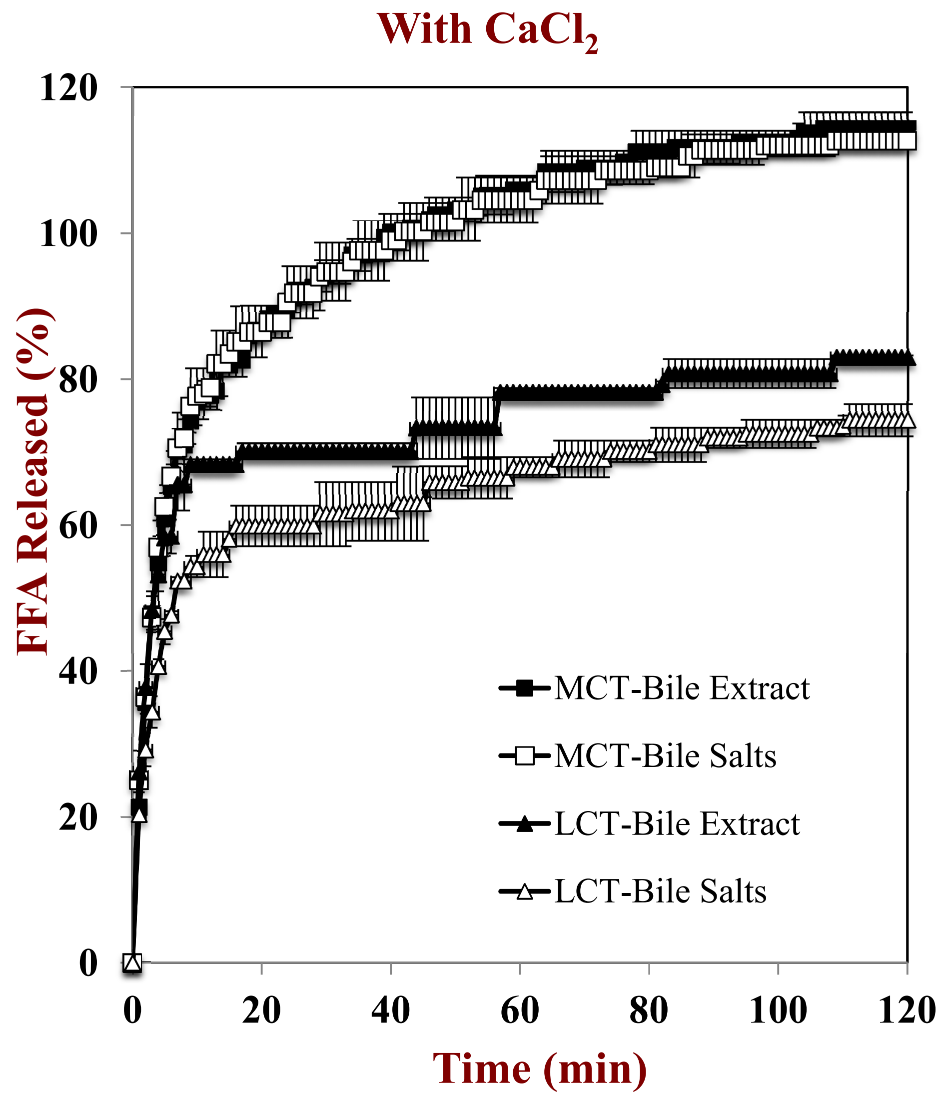


Figure 4e Influence of carrier oil type and bile salt type on the rate and extent of lipid digestion in vitamin E fortified emulsions measured using a pH-stat method. The SSIFs all contained calcium. Data are means±SD for n = 4 independent simulated digestions.

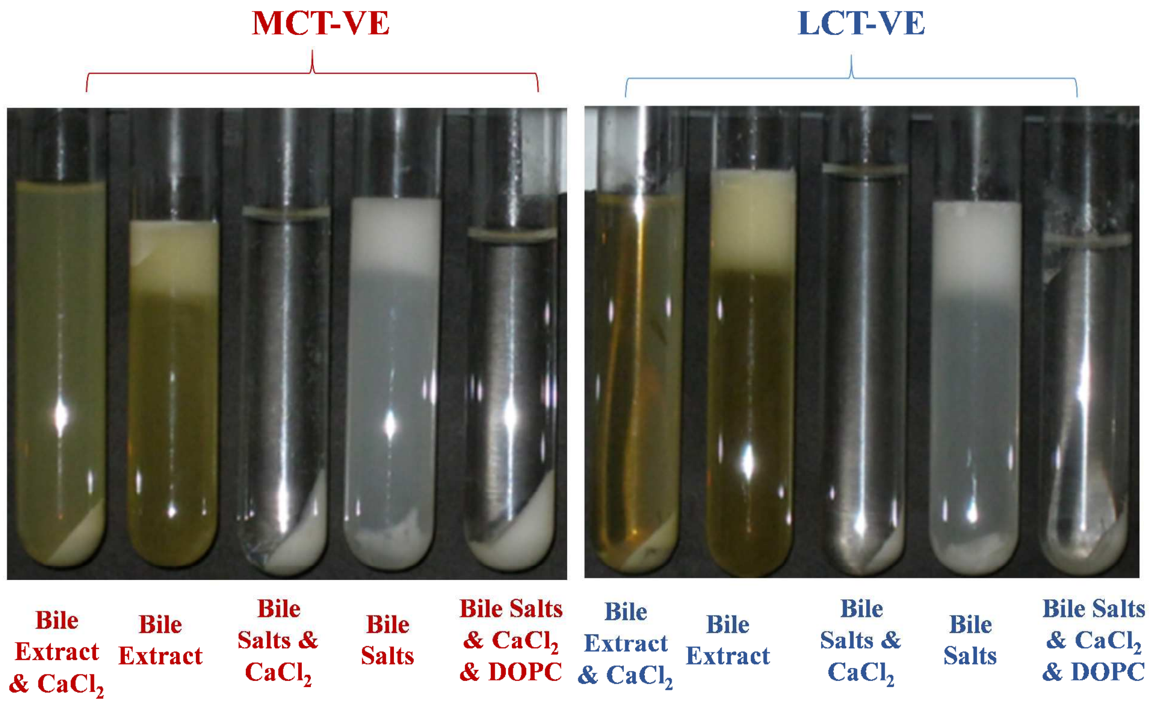


Figure 5. Influence of carrier oil type and SSIF composition on the appearance of the micelle phase after *in vitro* digestion of vitamin E fortified emulsions.

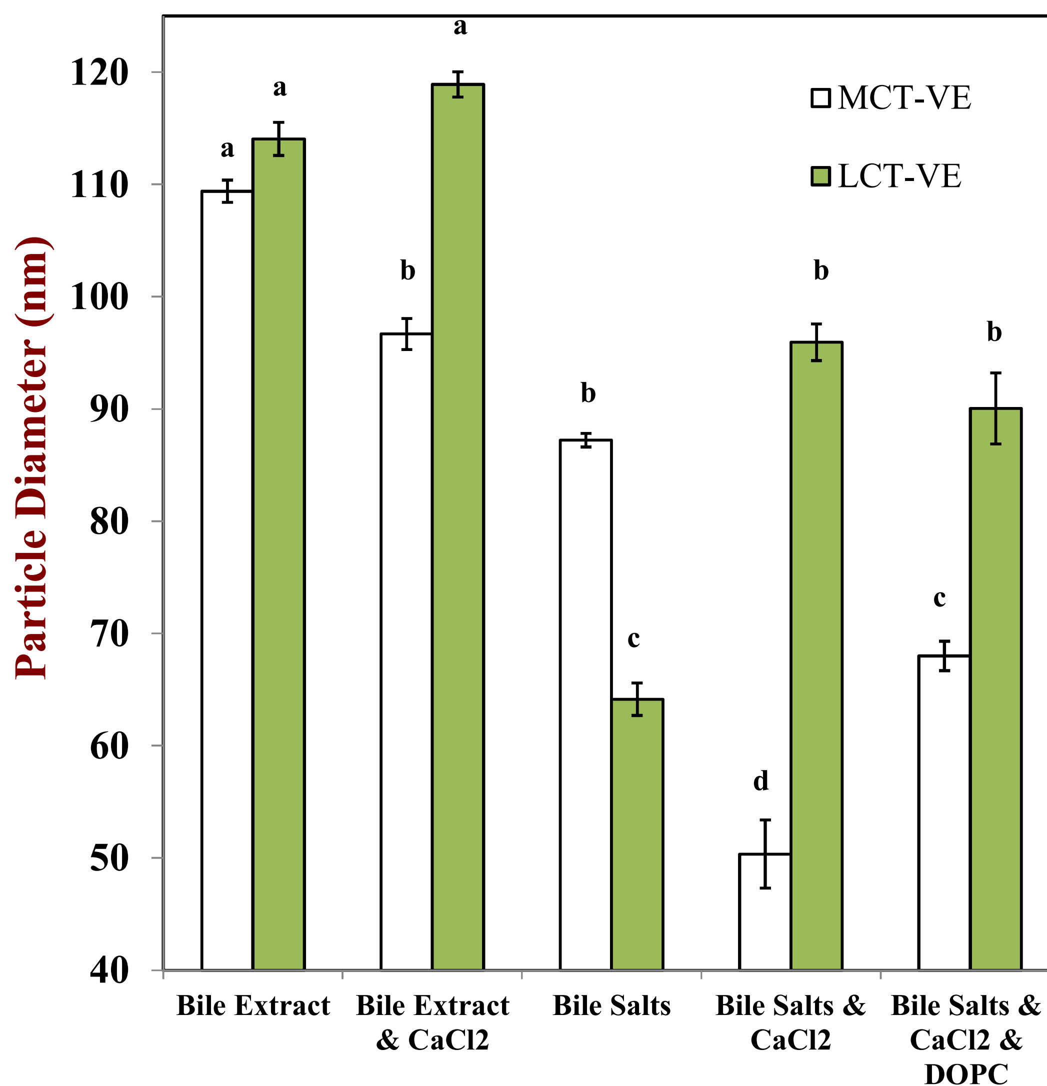


Figure 6. Influence of carrier oil type and SSIF composition on the mean particle diameter of the micelle phase after *in vitro* digestion of vitamin E fortified emulsions. The samples were filtered using a 450 nm filter prior to analysis to simulate passage through the mucus layer. Data are means ± SD for n = 4 independent simulated digestions. Letters show samples with significant differences ($p < 0.01$)

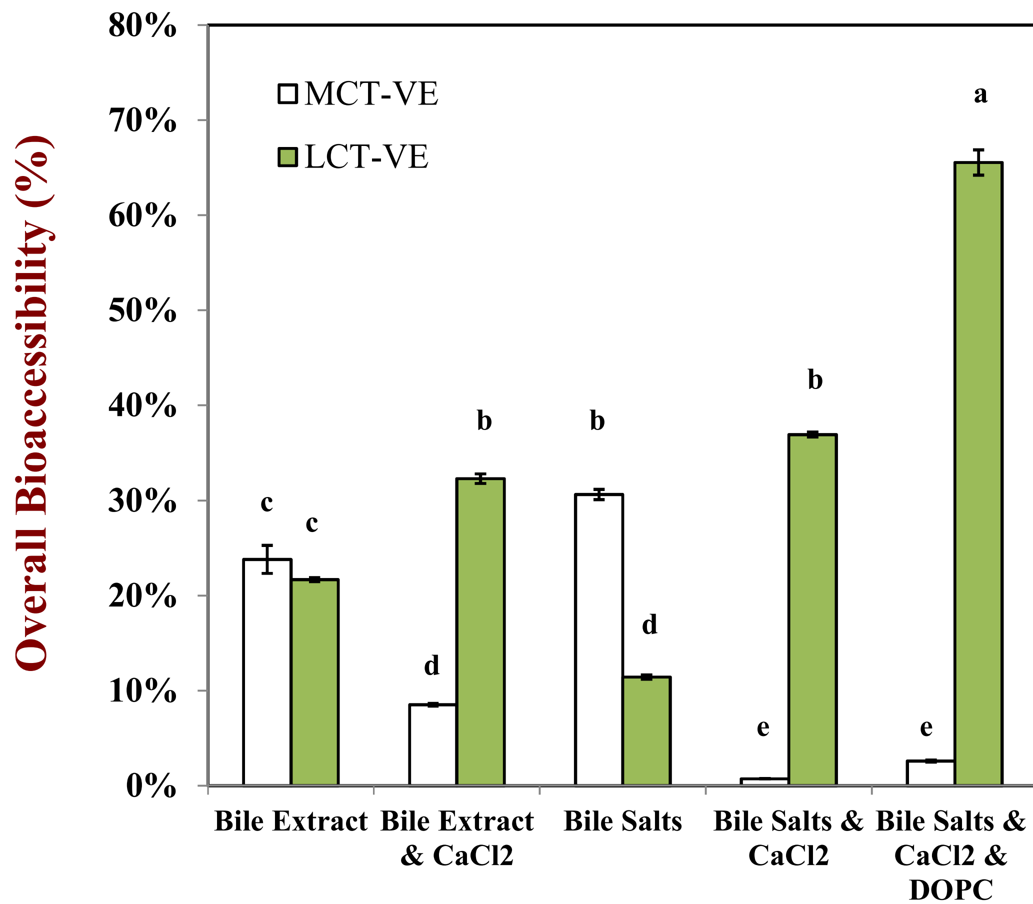


Figure 7. Influence of carrier oil type and simulated small intestine fluid composition on the overall bioaccessibility of Vitamin E (α-tocopherol + α-tocopherol acetate) in emulsion-based delivery systems after digestion. Data are means ± SD for n = 4 independent simulated digestions. Letters show samples with significant differences ($p < 0.01$).

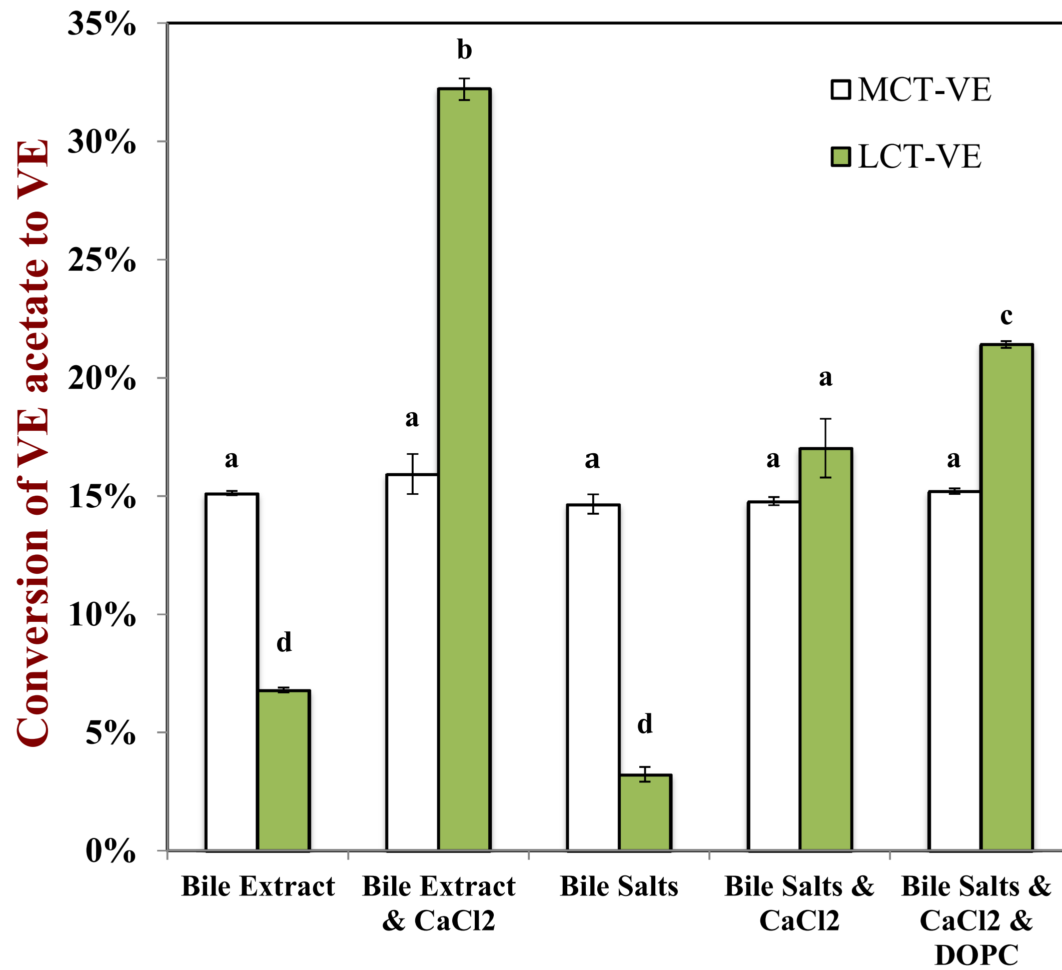


Figure 8. Influence of carrier oil type and SSIF composition on the conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion of vitamin E fortified emulsions. Data are means ± SD for n = 4 independent simulated digestions. Letters show samples with significant differences ($p < 0.01$).

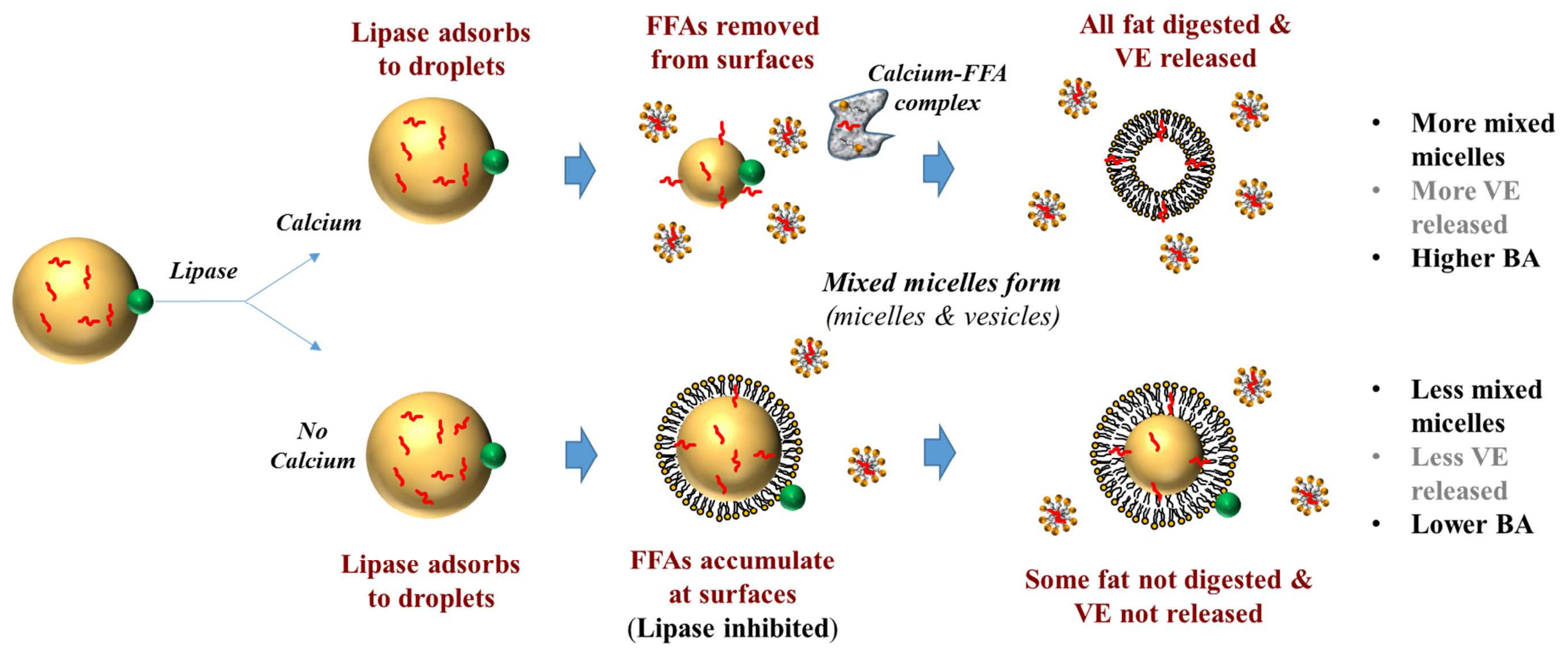


Figure 9a. Schematic representation of influence of calcium on physicochemical phenomena occurring within gastrointestinal tract during lipid digestion, vitamin release, and solubilization.

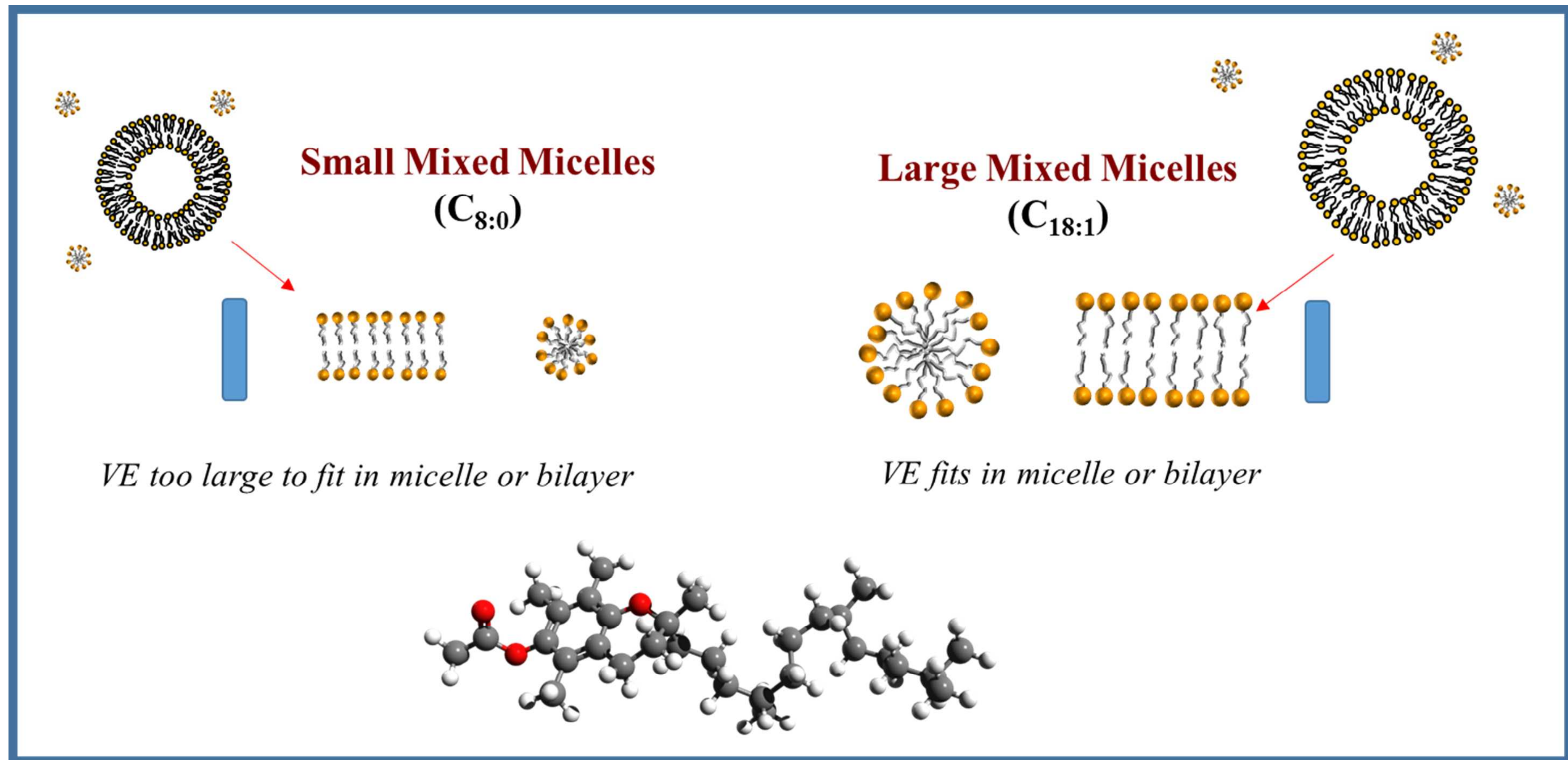


Figure 9b. Schematic illustration of the influence of free fatty acid chain length on the bioaccessibility of vitamin E. Long chain FFAs form mixed micelles that can easily accommodate large VE molecules, whereas mediuc chain FFAs do not.

Table 1: Compositions of the simulated small intestinal fluids (SSIFs) used in the study: NaC = sodium cholate; NaDC = sodium deoxycholate; DOPC = 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine (phospholipid). The solutions were all dissolved in PBS buffer (10 mM, pH 7).

SSIF	Bile Extract	NaC	NaDC	DOPC	$CaCl_2$	Lipase
Bile Extract	4 mL (187.5 mg)	0	0	0	0	2.5 mL (60 mg)
Bile Extract & Ca^{2+}	4 mL (187.5 mg)	0	0	0	1m L (110 mg)	2.5 mL (60 mg)
Bile Salts	0	4 mL (73.22 mg NaC and 114.06 mg NaDC)		0	0	2.5 mL (60 mg)
Bile Salts & Ca^{2+}	0	4 mL (73.22 mg NaC and 114.06 mg NaDC)		0	1m L (110 mg)	2.5 mL (60 mg)
Bile Salts & Ca^{2+} & DOPC	0	4 mL (73.22 mg NaC, 114.06 mg NaDC and 36 mg DOPC)			1m L (110 mg)	2.5 mL (60 mg)