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Factors impacting lipid digestion and nutraceutical bioaccessibility assessed by standardized gastrointestinal model (INFOGEST): Oil droplet size

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Abstract

The oil droplets in commercial emulsified foods have dimensions that vary widely, from hundreds of nanometers to tens of micrometers. Previously, the size of the droplets in oil-in-water emulsions has been shown to impact their gastrointestinal behavior, which may influence their physiological effects. In this study, we analyzed the impact of oil droplet diameter (0.16, 1.1 and 8.2 μm) on lipid digestion and nutraceutical bioaccessibility using a widely used standardized gastrointestinal tract model: the INFOGEST method. The emulsions used consisted of corn oil droplets stabilized using a food-grade non-ionic surfactant (Tween 20), and the droplet size was controlled by preparing them with a microfluidizer (small), sonicator (medium), or high-shear blender (large). The surfactant-coated oil droplets were relatively resistant to size changes in the mouth and stomach, due to the strong surface activity and steric stabilization mechanism of the non-ionic surfactant used. As expected, the kinetics of lipid digestion were enhanced for smaller droplets because of their greater specific surface area. The degree of lipid digestion fell from 117% to 78% ($p < 0.001$) as the initial droplet diameter was raised from 0.16 to 8.2 μm. In addition, there was a reduction in β-carotene bioaccessibility from 83 to 15% ($p < 0.001$) with increasing droplet diameter. This result was ascribed to several effects: (i) some carotenoids were trapped inside the undigested oil phase; (ii) fewer mixed micelles were produced to internalize the carotenoids; and, (iii) a fraction of the carotenoids crystallized and sedimented. Our results underline the critical importance of considering droplet size when developing emulsified foods loaded with carotenoids. The results obtained by the INFOGEST method are consistent with those found using other *in vitro* methods in earlier studies.

Keywords: Oil droplet size; β-carotene; emulsion; bioaccessibility; INFOGEST method.

1. Introduction

Many food products are colloidal systems consisting of tiny oil droplets distributed throughout an aqueous medium, such as milk, cream, soft drinks, mayonnaise, dressings, sauces, and toppings.[1] The oil droplet size in these products varies greatly due to different ingredients and processing operations used in their creation.[2-4] For instance, the mean droplet diameter is only a few hundred nanometers in soft drinks and homogenized milk, but tens of micrometers in dressings and mayonnaise, which corresponds to a three-orders of magnitude difference.[5, 6] The size of the droplets in an emulsified food product impacts it's appearance, rheology, flavor release profile, physical stability, and chemical stability. Consequently, each product must be carefully designed to have a particle size distribution that provides the required quality attributes and shelf-life for the particular application.

Emulsion droplet dimensions also influence the behavior of food products within the human gut,[7] which can have important nutritional and health implications. Studies using *in vitro* digestion models demonstrate that droplet dimensions influence lipid hydrolysis kinetics and nutraceutical bioaccessibility.[8-12] Typically, these studies demonstrate that smaller droplets are digested faster and more fully than larger droplets, mainly because the lipase molecules have more surface area per unit volume of oil to attach to. Moreover, the bioaccessibility of non-polar substances present in the oil phase of emulsions, such as hydrophobic nutraceuticals or vitamins, usually increases as the oil droplets become smaller. This bioaccessibility enhancement is attributed to the fact that more of the bioactives are liberated from the oil droplets and more mixed micelles are formed to solubilize them when the lipid phase is digested faster and more extensively.[7] Overall, these results demonstrate that emulsions with ultrafine droplets are more suitable in applications where rapid release and/or high bioavailability of a bioactive agent are

required.[13-16] These *in vitro* results are consistent with *in vivo* animal studies that have also demonstrated the oral bioavailability and absorption rate of hydrophobic bioactives increase when they are encapsulated in very small lipid droplets.[17-19]

Globally, there are numerous research groups developing emulsion-based delivery systems for various kinds of hydrophobic bioactive agents. Many of these researchers use different *in vitro* gastrointestinal models to assess the potential efficacy of their formulations. These *in vitro* models differ in the types and amounts of enzymes, minerals, bile salts, and minerals they contain, which makes direct comparisons difficult. For this reason, a team of international researchers developed a standardized simulated gastrointestinal tract (GIT) model, known as the INFOGEST method, which has been widely adopted in the field.[20, 21] This model has already been used to study lipid digestion and/or bioactive bioavailability in various emulsion systems.[22, 23] For instance, our group recently showed that it could be used to study the impact of calcium on carotenoid bioaccessibility [24] and chitosan on vitamin D bioaccessibility.[22] Other researchers have used it to study the influence of mayonnaise on the bioaccessibility of carotenoids in fruits.[25]

Our objective in this study was to establish the impact of oil droplet size (100 nm to 10 μm) on oil phase hydrolysis and nutraceutical bioaccessibility in model food emulsions using the INFOGEST method.[20] A carotenoid (β-carotene) was used in this study as a model of a strongly hydrophobic nutraceutical. Results using earlier (non-standardized) *in vitro* gastrointestinal models have shown that oil phase hydrolysis and carotenoid bioaccessibility increase as the droplet dimensions are reduced.[9, 12] These models use different GIT conditions (such as bile salt, calcium, and enzyme levels) than the harmonized INFOGEST model. For this reason, we wanted to determine whether the results obtained using the INFOGEST model were consistent with

93 those obtained using these earlier *in vitro* models. Based on previous results, we hypothesized
94 that oil phase hydrolysis and carotenoid bioaccessibility would still increase as the droplet size
95 was decreased, but the magnitude of this effect was unknown. The knowledge gained through
96 this research on the impact of oil droplet size should enrich our understanding of the impact of
97 food matrix effects on the biological activity of hydrophobic nutraceuticals, as well as providing
98 insights into the differences between gastrointestinal models. It should be noted that this study is
99 part of a series where we are using the INFOGEST method to systematically examine the impact
100 of key factors on the gastrointestinal fate of emulsified foods, such as oil droplet concentration
101 and emulsifier type.[26, 27] The aim of these studies is to provide some fundamental insights into
102 the major factors impacting the gastrointestinal behavior of more complex real food systems.
103

2. Materials and methods

2.1. Materials

Corn oil (Mazola, ACH Food Companies, Memphis, TN, USA) was obtained from a local store. Tween 20 was purchased from ACROS Organic (Pittsburgh, PA, USA). Chemicals purchased from the Sigma-Aldrich Company (St. Louis, MO, USA) included: β-carotene (Type Ⅰ, synthetic, ≥93% in UV); porcine gastric mucin; pepsin from porcine gastric mucosa (250 units/mg, P7000); pancreatin from porcine pancreas (P7545); porcine lipase (100-400 units/mg, P3126); and, porcine bile extract. Information about the methods used to measure the activity of these different enzymes are given in the supplier's website (www.sigmaaldrich.com). Ethyl alcohol (ACS/USP grade) was obtained from Pharmco Products, Inc. (Shelbyville, KY, USA). All other chemicals and reagents (analytical grade or higher) were purchased from either Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA, USA). All solutions and emulsions were prepared using double distilled water obtained from a water-purification system (Nanopure Infinity, Barnstaeas International, Dubuque, IA, USA).

2.2. Preparation of emulsion-based delivery systems

Carotenoid-fortified emulsions were fabricated according to a method we have used before.[28] An aqueous phase was produced by dissolving non-ionic surfactant (0.5% Tween 20, w/w) in phosphate buffer solution (5 mM, pH 7.0). The oil phase was produced by dissolving β-carotene (0.1%, w/w) in warmed corn oil (50 ºC) with sonication and stirring. The oil phase (5%, w/w) and aqueous phase (95%, w/w) were mixed together using different homogenization methods to prepare emulsions containing different-sized droplets. Emulsions with large-sized droplets ("large emulsion") were prepared by a high-shear blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) at 10,000 rpm, for 6 min. Emulsions with medium-sized

droplets ("medium emulsion") were prepared by sonicating a portion of the large emulsion (Sonicator FB505, Thermo Fisher Scientific, Waltham, MA, USA). The sonication conditions used were as follows: diameter of tip probe = 13 mm, bottom gap = 10 mm, frequency = 20 kHz, power = 500 W, amplitude = 20%, sonication on/off duration = 2/2 s, total sonication time = 3 min. An emulsion containing small oil droplets ("fine emulsion") was prepared by microfluidizing a portion of the large emulsion (M110Y, Microfluidics, Newton, MA) at 12000 psi for 3 circulations.

2.3. Droplet size, charge, and microstructure

The size, charge, and spatial location of the particles in the samples was carried out according to our recent study.[22] Mean particle diameters ($D_{3,2}$) and particle size distributions of initial and digested emulsions were measured using static light scattering (Mastersizer 2000, Malvern Instruments, Malvern, Worcestershire, UK). Mean particle diameters (Z-average) of mixed micelle samples were measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments). Surface potential (ζ-potential) values of the particles in all samples were measured by microelectrophoresis (Zetasizer Nano ZS, Malvern Instruments). Microstructures of lipid-stained (Nile red) samples were collected using confocal fluorescent microscopy (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, USA).

2.4. *In vitro* digestion

In vitro digestion of carotenoid-loaded emulsions was performed using the recently updated harmonized INFOGEST method,[20] with slight adaptations: mucin was added to the mouth phase; gastric lipase was omitted from the stomach phase; and a pH stat method was used to monitor lipid digestion in the small intestine phase. Briefly, emulsions were exposed to simulated oral, gastric, and intestinal phases containing the appropriate GIT components and with the

appropriate pH values, stirring rates (100 rpm), and incubation times (37 °C). Free fatty acid release during lipid digestion in the small intestinal phase was monitored using the pH stat method.[24] The intestinal samples were centrifuged (Sorvall Lynx 4000 centrifuge, Thermo Scientific, Waltham, MA, USA) at 46,285 ×g (18,000 rpm) at 4 °C for 50 min to separate the mixed micelle and sediment phases. In this study, gastric lipase was not included so we could focus on lipid digestion in the intestinal phase (where the majority of lipid digestion occurs) and use the simple pH stat method to monitor the impact of droplet size on digestion. In future, studies it would also be interesting to examine the impact of gastric lipase on the digestion of emulsified lipids with different droplet sizes, as this can make up an appreciable contribution to the total digestion in some systems.

2.5. Extraction and analysis of β-carotene

β-carotene was extracted from the digested samples and then analyzed using an established method [29] with slight modifications. Briefly, an organic solvent (2:3 v/v hexane/isopropanol) was used to extract the carotenoids. The β-carotene concentration was found by measuring the absorbance of the carotenoid-loaded organic phase at 450 nm using a UV-visible spectrophotometer (Genesys 150, Thermo Scientific, Waltham, MA, USA). Organic solutions of known β-carotene concentration were used to prepare the calibration curve ($R^2 = 0.9995$). The bioaccessibility, release, and stability (%) of the β-carotene were calculated using the following equations:

$$\text{Bioaccessiblity} = 100 \times \frac{C_{micelle}}{C_{digesta}}$$

$$\text{Release} = 100 \times \frac{C_{micelle} + C_{sediment}}{C_{digesta}}$$

$$\text{Stability} = 100 \times \frac{C_{digesta} \times \text{DF}}{C_{initial}}$$

Here, $C_{micelle}$, $C_{sediment,}$ $C_{digesta,}$ and $C_{initial}$ are the concentrations of β-carotene in samples collected from the mixed micelle, sediment, total intestine digesta, and initial emulsion, respectively. Also, DF is the dilution factor for the gastrointestinal experiments (= 8).

2.6. Statistical analysis

Emulsions were prepared in duplicate, and the digestion process and other characterization assays were performed in triplicate. Means and standard deviations were then calculated. The statistical differences among samples were calculated at a confidence level of 95% using ANOVA with either Tukey test (homogenous) or Dunnett's T3 test (inhomogeneous). SPSS software (IBM Corp., Armonk, NY, USA) was used to perform all statistical calculations.

3. Results and discussion

3.1. Structural and physical properties in simulated gastrointestinal tract

In this study, the initial emulsion compositions were fixed as 0.005% β-carotene, 5.0% corn oil, 0.5% Tween 20, and 94.5% phosphate buffer solution (pH 7, 5 mM). This surfactant is known to be a good emulsifier because it rapidly adsorbs to oil-water interfaces, reduces the interfacial tension appreciably, and forms a steric barrier.[30] Emulsions with a range of different target average particle diameters (≈ 0.1, 1 and 10 μm) were prepared using a microfluidizer, sonicator, and blender respectively. The actual measured $D_{3,2}$ values of these emulsions were 0.158, 1.09 and 8.20 μm respectively (Fig. 1a). For clarity and concision, these samples are called "fine", "medium" and "large" emulsions in the following discussion. The particle size distributions of all the initial emulsions were roughly monomodal (Fig. 2a). The microscopy analysis indicated a similar general trend in particle size with homogenization conditions (Fig. 3).

With increasing oil droplet size, more creaming occurred in the emulsions when they were left to stand under quiescent conditions for 24 hours (Fig. 4a). This phenomenon is expected since the gravitational force operating on an individual oil droplet is proportional to the square of its diameter.[6] Hence, larger droplets should move upwards much more quickly than smaller ones, which would influence the storage stability and shelf life of commercial products.[31]

The surfactant-coated oil droplets all had negative surface potentials (-23.9 to -18.0 mV) (Fig. 1b). Tween 20 is supposed to be a non-ionic surfactant and so the negative charge may arise from other anionic species present at the oil droplet surfaces, *e.g.,* hydroxyl ions or free fatty acids.[6] This result suggests that the oil droplets may be stabilized by both steric and electrostatic repulsive forces.

The different-sized emulsions were then passed through the INFOGEST model to understand their gastrointestinal fate. The physical and structural properties of samples were analyzed at the end of the sequential stages of this digestion model. In addition, they were measured at the start of the small intestine ("SI-initial"). We carried this out by taking the emulsions collected from the end of the gastric phase and then adjusting them to pH 7. Their properties were then measured before introducing the bile salts and digestive enzymes. This procedure was carried out because the aggregation state of oil droplets entering the small intestine impacts their subsequent digestion.[32-34]

In the mouth, stomach, and SI-initial phases, the oil droplet size in all emulsions remained approximately the same as those in the initial emulsions (Figs. 1a and 3). Thus, the Tween 20-coated oil droplets were resistant to aggregation and disruption in the early stages of the INFOGEST model irrespective of their initial size. Tween 20 has a high surface-activity so it attaches strongly to droplet surfaces and is difficult to displace. Moreover, it generates strong steric repulsive forces that prevent droplets from coming together and aggregating. In contrast, protein- or phospholipid-coated oil droplets often become aggregated under mouth or stomach conditions because of the reduction in electrostatic repulsive forces operating between them.[35, 36] In addition, the non-ionic head groups of Tween 20 mean that it is difficult for mucin to adsorb to the droplet surfaces in the mouth and stomach phases.

The droplets in all the emulsions were strongly negative (-26.0 to -16.7 mV) when they were dispersed in neutral pH solutions, such as those present in the initial emulsions, oral, and SI-initial phases. Conversely, they were only weakly negative (-1.9 to -1.7 mV) under the acidic solution conditions in the gastric phase (Fig. 1b). Interestingly, the surface potential of the oil droplets did not depend on their size. The reduced negative charge of the surfactant-coated oil

droplets in the stomach phase is most likely due to the protonation of free fatty acid impurities or the reduced adsorption of hydroxyl ions from the water under acidic conditions.

After intestinal digestion in the presence of lipase, the physical and structural properties of all the emulsions changed considerably. A significant ($p < 0.05$) increase in the average size of the particles in the fine emulsions was observed (Fig. 1a), as well as evidence for a wide range of different-sized particles in the samples (Fig. 2b). On the other hand, the average size of the particles in the medium and large emulsions significantly ($p < 0.05$) decreased, due to the presence of a substantial fraction of small particles (< 1 μm) in the particle size distribution after digestion (Figs.1a and 2b). During the intestinal phase, the triglycerides inside the oil droplets are hydrolyzed to fatty acids and monoglycerides through a hydrolysis reaction. These lipid digestion products then interact with constituents within the gastrointestinal fluids (such as calcium, bile salts, and enzymes) to form a range of differently-sized colloidal assemblies, *e.g.,* micelles, vesicles, liquid crystals, aggregated proteins, and fatty acid/calcium soaps.[37, 38] Electron microscopy and light scattering methods have shown that most of the colloidal particles present in the digest are smaller than about 1000 nm, such as spherical micelles (up to 10 nm), vesicles (up to 100 nm) and multivesicular liposomes (up to 1000 nm).[33, 37] These colloidal particles are therefore larger than the oil droplets in the initial fine emulsions, but smaller than those in the medium and large emulsions. Similar size changes after intestinal digestion have also been noted in whey protein-stabilized emulsions.[35] The mean diameters of the particles remaining within the intestinal fluids after digestion were 0.364, 0.410, and 0.825 μm for the fine, medium, and large emulsions, respectively (Fig. 1a). Conversely, the Z-average values of the micelle samples (collected by centrifugation) were similar for all samples, being 195, 200, and 202 nm for fine, medium, and large emulsions, respectively (Fig. 5). This suggests that some of the larger and

251 denser colloidal particles formed during lipid digestion, such as calcium soaps and/or large
252 multivesicular liposomes, may have been at least partially removed by centrifugation.
253 Our results suggest there were some undigested lipid droplets within the small intestine
254 phase collected from the larger emulsions. Indeed, the confocal microscopy images and visual
255 appearance of the samples showed numerous large undigested oil droplets in the large emulsions,
256 as well as several smaller undigested oil droplets in the medium emulsions (Figs 3 and 4b). The
257 results of the INFOGEST method are therefore consistent with those obtained with other *in vitro*
258 digestion methods, where researchers also reported some undigested oil phase in emulsions
259 containing relatively large oil droplets.[8, 39] The fine and medium emulsions appeared much more
260 turbid than the large emulsions after digestion under small intestine conditions (Fig. 4a). This
261 suggests that they contained more sub-micron particles that scattered light strongly.
262 The absolute value of the negative ζ-potential of all the emulsions increased significantly (p
263 < 0.05) after intestinal digestion (Fig 1b), which is probably due to the generation of anionic fatty
264 acids. Interestingly, the absolute value of the surface charge was similar for the fine and medium
265 emulsions but significantly ($p < 0.05$) low for the large emulsions (Fig 1b). This is probably
266 because the large oil droplets were not fully digested (see next section), and so less anionic fatty
267 acids were generated.

268 **3.2. Lipid digestion in the intestinal digestion**

269 The production of free fatty acids (FFAs) during the intestinal phase was followed using a
270 pH stat method. Initially, the volume of alkaline titrant needed to neutralize the FFAs produced
271 during digestion was continuously recorded. However, a fraction of the FFAs released during
272 lipid digestion from long chain triglycerides (like those in corn oil) are not ionized at pH 7, so
273 they are not titrated by NaOH during pH stat measurements.[40, 41] Therefore, after digestion was

completed, a back-titration was performed to pH 9 to determine the total fraction of FFAs released by the various emulsions: 117, 113 and 78% for the fine, medium, and large emulsions, respectively (Fig. 6a). The INFOGEST method uses relatively high enzyme levels to simulate fed conditions in the human gut, which would account for the high degree of lipid digestion observed in the emulsions after back titration. It should be noted that these values are considerably higher than the total fraction of FFAs calculated without the back-titration for the same emulsions: 60, 52, 37%, respectively (Fig. 6a). Again, this difference indicates that not all of the free fatty acids were fully ionized under neutral small intestine conditions, and so they are not titrated by the alkaline solution at pH 7. For this reason, a correction factor (CF) was employed to determine the actual level of FFAs produced during lipid hydrolysis. The correction factor was calculated as: CF = Final FFAs (pH 9)/Final FFAs (pH 7).

The kinetics of lipid digestion clearly depended on the size of the oil droplets in the emulsions entering the small intestine (Fig. 6b). For all samples, the percentage of FFAs produced increased rapidly during the initial stages and then more slowly later. Nevertheless, the initial rate of FFAs released became faster as the droplet size was reduced, and the total amount of fatty acids released by the end of digestion was appreciably higher for the small and medium emulsions than the large emulsions. The results of the INFOGEST method are therefore consistent with those found by previous researchers using simpler *in vitro* digestion models.[8, 9, 12, 39] This effect occurs because the specific surface area (A_S) of the oil droplets in an emulsion is inversely proportional to their average diameter ($D_{3,2}$). Consequently, there is more lipid surface available for the lipase molecules to attach to for emulsions containing smaller droplets. The initial lipid digestion rates calculated from the free fatty acid release profiles (first 5 minutes) were 21.1, 15.2, and 7.0 FFA/min for the small, medium, and large emulsions, respectively. This

297 suggests that there was a positive, though not direct, correlation between the lipid digestion rate
298 and the droplet surface area.

299 Interestingly, the total fraction of FFAs produced by the final stages of lipid digestion
300 exceeded 100% for the small and medium emulsions (Fig. 6b). The calculation of the percentage
301 of FFAs released using the pH stat method is based on the assumption that only two FFAs and
302 one monoglyceride are generated per triglyceride molecule due to the action of pancreatic
303 lipase.[7] In practice, some of the monoglycerides may be further hydrolyzed into a glycerol
304 molecule and another free fatty acid, thereby leading to values over 100%. Indeed, previous
305 researchers have shown experimentally that monoglycerides can be degraded through this
306 mechanism.[9, 42]

307 **3.3. Stability, release, and bioaccessibility of β-carotene**

308 The bioaccessibility of hydrophobic nutraceuticals trapped inside oil droplets is known to
309 depend on the digestion of the surrounding lipid phase.[7] Consequently, we measured the
310 influence of oil droplet size on the bioaccessibility of the β-carotene in the emulsions. The
311 carotenoid concentration of the initial emulsions was measured, as well as in various fractions
312 collected after small intestinal digestion (sediment phase, micelle phase, total digested sample).
313 The stability, release, and bioaccessibility of the β-carotene were then calculated from these
314 values.

315 *Carotenoid stability:* β-carotene stability in the samples to degradation and/or loss as they
316 passed through the simulated GIT was defined as the total concentration measured in the small
317 intestine divided by that measured in the initial emulsion. The stability of the β-carotene was
318 significantly ($p < 0.05$) higher in the fine and medium emulsions (75%) than in the large
319 emulsions (65%) (Fig. 7a). β-carotene is susceptible to chemical degradation when exposed to

heat, light, or acidic conditions.[43] Consequently, some of the β-carotene may have degraded within the acidic gastric environment (pH 3), especially since it was held there for two hours at a slightly elevated temperature (37 ^{o}C). In addition, some of the β-carotene-loaded oil droplets may have adhered to the sides of the containers used to hold or transfer the emulsions within the simulated GIT, and so were not detected in the small intestine. Typically, the attractive forces between colloidal particles and surfaces increase as the particle size increases,[44] which may have led to more of the larger droplets being lost through this mechanism.

Carotenoid release: The fraction of β-carotene released by the oil droplets was calculated as the sum of the concentrations measured in the sediment and micelle phases divided by the total concentration measured in the small intestine phase after digestion. We assumed that any non-released β-carotene was still associated with the oil phase (which formed a thin surface layer on some emulsions). Carotenoid release decreased significantly ($p < 0.05$) as the oil droplets became larger, being 94.7, 76.0 and 55.0% for the fine, medium, and large emulsions, respectively (Fig. 7a). We attributed this effect to the reduction in lipid digestion as the droplets became bigger, which is supported by the lower level of free fatty acids produced during digestion (Fig. 6), the appearance of a thin surface layer on medium and large emulsions (Fig. 4b), and the existence of large non-digested oil droplets in the microscopy results (Fig. 3). It should be noted that this non-released fraction would be expected to reduce the bioaccessibility of the carotenoids in the small intestine phase. However, it is possible that any β-carotene remaining in the oil phase could travel to the colon and be released there, provided there are digestive enzymes available to break down the lipid phase. Moreover, this feature could be beneficial for the creation of emulsion delivery systems with extended release profiles for

342 hydrophobic nutraceuticals. However, further experiments, both *in vitro* and *in vivo*, are needed
343 to test these hypotheses.

344 *Carotenoid bioaccessibility:* β-carotene bioaccessibility was calculated as the concentration
345 measured in the micelle phase divided by the total concentration measured in the digested
346 samples after the small intestine phase. After being released from the oil droplets, some of the β-
347 carotene is incorporated into the mixed micelles in the aqueous phase that are typically formed
348 from monoglycerides, free fatty acids, bile salts, and phospholipids. It is typically assumed that
349 only β-carotene in this form can travel through the mucus layer and be internalized by the
350 epithelium cells.[45]

351 In our study, the percentage of β-carotene in a bioaccessible form decreased significantly (p
352 < 0.05) with increasing droplet size, being 82.5, 46.5, and 15.0% for the fine, medium, and large
353 emulsions, respectively (Fig. 7b). Conversely, the percentage of β-carotene within the sediment
354 phase increased significantly ($p < 0.05$) with increasing droplet size, being 12.2, 29.5, and 44.4%
355 for the corresponding emulsions. The same trends were observed in the measurements of the
356 absolute concentrations of β-carotene in the micelle and sediment phases (Fig. 7c). Specifically,
357 the carotenoid concentration in the micelles decreased significantly ($p < 0.05$) as the droplet size
358 increased, changing from 3.17 μg/ml for the fine emulsion to 0.57 μg/ml for the large emulsion.
359 At the same time, the carotenoid concentration in the sediment phase increased with increasing
360 droplet size, changing from 0.43 μg/ml for the fine emulsion to 1.82 μg/ml for the large
361 emulsion. On the other hand, the total concentration of carotenoids in the overall small intestine
362 phase remained fairly constant (3.9 to 4.8 μg/ml).

363 The high bioaccessibility and low sedimentation of the fine emulsions are attributed to
364 complete lipid digestion, high micellization, and low precipitation of the carotenoids under the

digestion conditions used in the INFOGEST method *i.e.,* high lipase, high bile salts, and low calcium). Conversely, our results suggest that the lower β-carotene bioaccessibility observed for larger droplets is due to two main factors: (i) some of the carotenoids was not released from the oil phase because it was not fully digested (Fig. 7a); (ii) some of the carotenoids that were released from the oil droplets precipitated and were therefore incorporated into the sediment phase (Fig. 7c). The fraction of non-digested oil was shown earlier to increase when the droplet size was reduced (Fig. 6), which would account for more of the carotenoids remaining within the oil phase at the end of digestion (Factor (i)). There are two potential causes for more precipitation of the β-carotene in emulsions containing larger oil droplets (Factor (ii)). First, it might be due to differences in the relative rates of lipid digestion, carotenoid release, carotenoid solubilization, and carotenoid crystallization.[46] In relatively large droplets, which are only digested slowly, the release and solubilization of carotenoids are also relatively slow, so they tend to accumulate inside the oil droplets. As a result, their concentration in the oil phase increases, until eventually it exceeds the saturation limit, and the carotenoids form water-insoluble crystals. Conversely, in relatively small droplets, which are digested rapidly, the carotenoids are quickly released and solubilized into the mixed micelles, which avoids the formation of large carotenoid crystals. A second reason may arise as a result of differences in the surface curvature of oil droplets with different dimensions, which leads to various types of colloidal particles being formed during lipid digestion. Large oil droplets have relatively low curvatures, thereby leading to the formation of larger vesicles at the oil droplet surfaces. Conversely, small oil droplets have relatively high curvatures, which may promote the formation of smaller micelles or vesicles at the droplet surfaces. Once formed, the larger vesicles may be more prone to precipitate due to their interactions with calcium ions in the gastrointestinal fluids,

388 thereby leading to the production of more calcium soap precipitates. However, more experiments
389 are clearly needed in this area to verify these hypotheses.

390 In this study, carotenoid bioaccessibility increased as the fraction of FFAs released from the
391 emulsions increased (Fig. 8). There was a gradual increase in bioaccessibility when the
392 percentage of FFAs released increased from 78% (large emulsion) to 113% (medium emulsion),
393 followed by a steep increase when the percentage of FFAs released increased to 117% (fine
394 emulsion). We hypothesize that this effect occurred because more and more β-carotene
395 accumulated within the oil phase as lipid digestion proceeded, because lipid digestion was faster
396 than carotenoid release.[46] Consequently, any non-digested oil may have contained quite high
397 levels of carotenoid. A layer of oil was clearly discernable on top of the medium and large
398 emulsions after digestion, but not in the fine emulsions. This effect would therefore account for
399 the large increase in bioaccessibility observed when moving from the medium to fine emulsions
400 (Fig. 8).

401 Overall, our results are consistent with earlier studies using non-standardized *in vitro*
402 digestion models, which have also reported a decrease in bioaccessibility with increasing droplet
403 size.[9, 10, 12] This suggests that results from the new harmonized INFOGEST method can be
404 compared to those obtained using these earlier *in vitro* digestion methods, at least qualitatively.
405 In a series of recent studies, we have systematically examined a number of food matrix effects
406 (oil droplet concentration, oil droplet size, and emulsifier type) on lipid digestion and carotenoid
407 bioaccessibility in emulsions using the INFOGEST method [26, 27]. Taken together, these studies
408 show that oil droplet size is one of the most critical factors influencing the gastrointestinal fate of
409 food emulsions, and that the impact of other factors (such as emulsifier type) can largely be
410 accounted for by their impact on the oil droplet size during digestion.

4. Conclusions

The impact of oil droplet size (0.1, 1 and 10 μm) on the bioaccessibility of β-carotene encapsulated within model food emulsions was characterized using the standardized INFOGEST digestion model. These particle sizes were selected to cover a broad range of oil droplet sizes found in commercial food products. During the digestion process, the surfactant-coated oil droplets were stable to aggregation or dissociation prior to adding the pancreatic lipase in the small intestine phase. After adding the lipase, the triglycerides inside the oil droplets were broken down into monoglycerides and free fatty acids at a rate depending on their size. The initial rate of lipid digestion and the final concentration of free fatty acids released increased with decreasing droplet size, which was attributed to the increase in surface area available for lipase to attach to. The suppression of lipid digestion in emulsions containing large droplets had a pronounced impact on carotenoid bioaccessibility. As the droplet size increased, the amount of β-carotene in the mixed micelles decreased, while that in the non-digested oil and sediment increased. The decrease in carotenoid bioaccessibility was mainly attributed to the fact that some of the carotenoids stayed within the non-digested oil droplets remaining after digestion of the large emulsions. Moreover, some of the carotenoids may have formed dense crystals that were trapped in the sediment phase. Overall, the results obtained using the standardized INFOGEST method were in good agreement with those obtained using earlier *in vitro* digestion methods.

The results from this study should contribute to the design of food products with tunable biological effects, such as prolonging satiety or nutraceutical blood levels by delaying lipid hydrolysis and nutraceutical release. Nevertheless, *in vivo* experiments are required to establish whether similar phenomena are observed in practice. Moreover, the impact of droplet size on the gastrointestinal fate of the emulsions is likely to depend on emulsifier type because this

434 determines their resistance to size changes within the mouth and stomach prior to reaching the
435 small intestine. In future studies, it will be important to investigate the impact of oil droplet size
436 on the gastrointestinal fate of real emulsified food systems, such as beverages, dressings, sauces,
437 dips, and desserts, as these are typically structurally and compositionally more complex than the
438 simple model systems used in this study. Moreover, it will also be important to compare the
439 results obtained with static *in vitro* methods (such as the INFOGEST one) with those obtained
440 using more realistic dynamic digestion models, as well as *in vivo* feeding trials using animals or
441 humans, to better understand the impact of oil droplet size on the gastrointestinal fate of foods.

442 # Conflicts of interest

443 There are no conflicts to declare.

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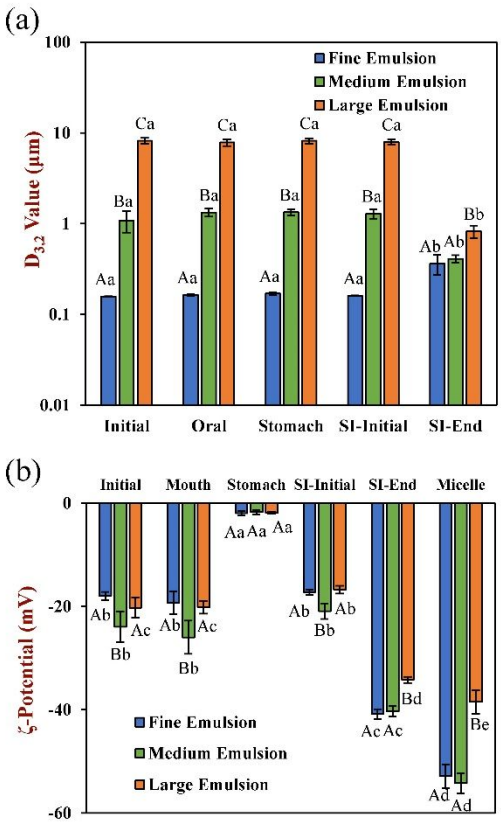


Fig. 1 The effect of oil droplet size on: (a) mean particle diameter ($D_{3,2}$) and (b) ζ-potential of the corn oil-in-water emulsions during *in vitro* gastrointestinal digestion. Different capital letters (A, B, C) were used to designate significant difference ($p < 0.05$) among different oil droplet size (same stage), and lower-case letter (a, b, c) for different stage (same oil droplet size). SI is used as an abbreviation for small intestine. Micelle indicates the mixed micelle phase (supernatant fraction) of the intestinal samples. Data is reported as mean ± SD (n=6).

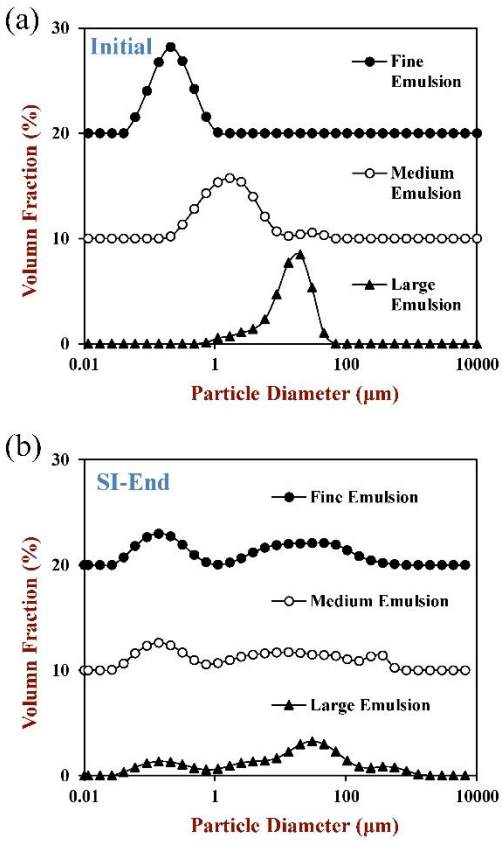


Fig. 2 The effect of oil droplet size on the particle size distribution of the corn oil-in-water emulsions (initial) and at the end of gastrointestinal digestion (SI-end). *Note*: the volume fraction was stacked up the y-axis for comparison (using an increment of 10%).

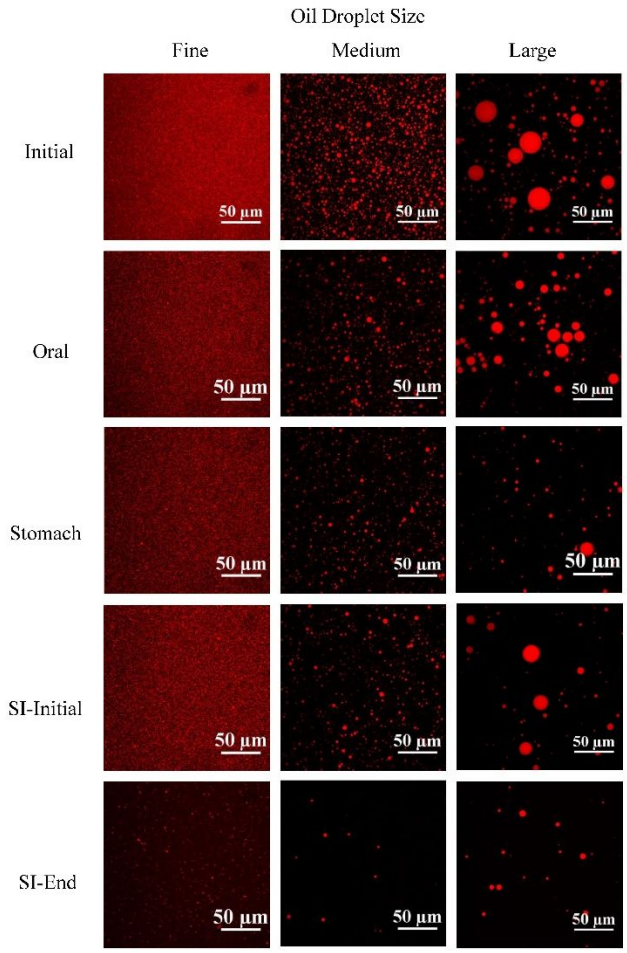


Fig. 3 The effect of oil droplet size on the confocal microscopy images of the corn oil-in-water emulsions during *in vitro* digestion. SI is used as an abbreviation for small intestine.

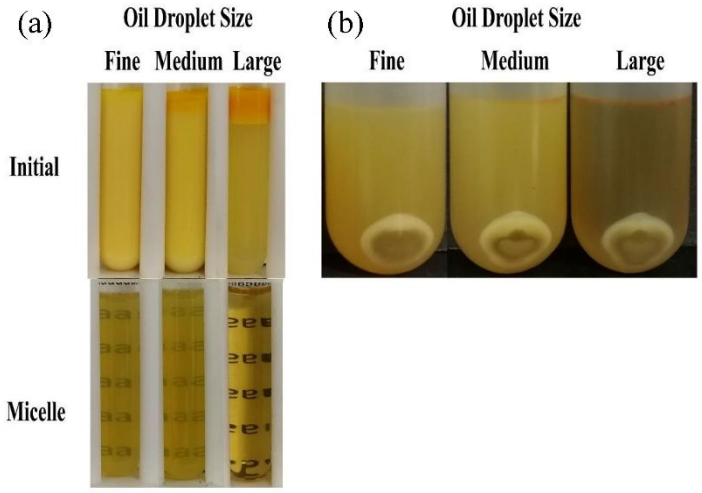


Fig. 4 The effect of oil droplet size on: (a) the appearance of the initial emulsions and the mixed micelle samples (supernatant fraction of the intestinal samples) collected after digestion; (b) the appearance of centrifugation separation of the emulsions after intestinal digestion (note the sediment at the bottom of the tubes).

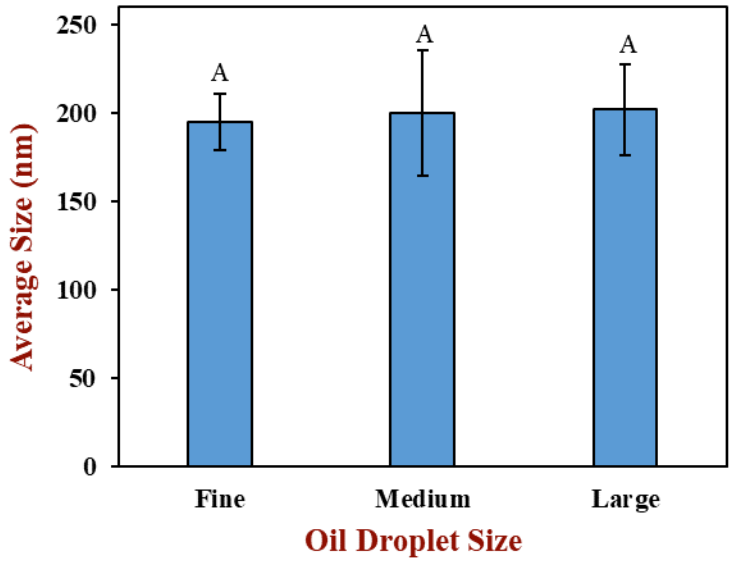


Fig. 5 The average diameter of the particles in mixed micelle samples (supernatant fraction of the intestinal samples) obtained after intestinal digestion of emulsions with different initial oil droplet sizes. Capital letters (A, B, C) were used to indicate significant difference ($p < 0.05$) among samples. Data is reported as mean ± SD (n=6).

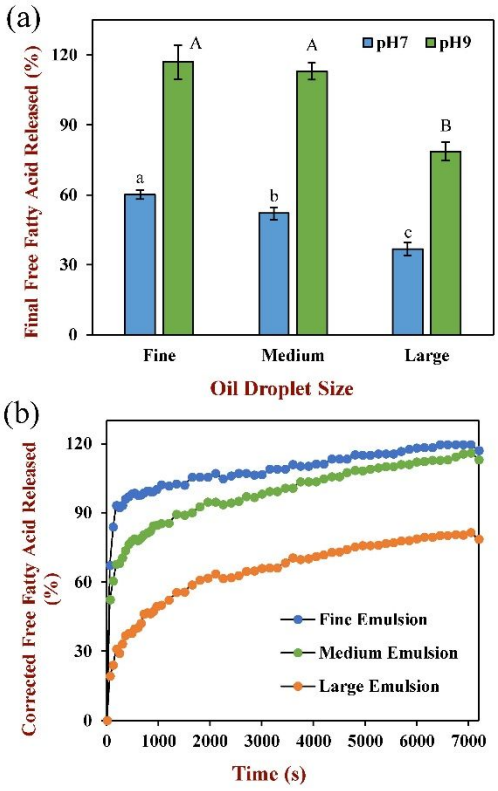


Fig. 6 The effect of oil droplet size on: (a) final free fatty acid (FFA) released; (b) corrected FFA released profile during small intestine digestion. Significant differences ($p < 0.05$) among samples with different oil droplet size in terms of the final FFA released at pH 7 and pH 9 were labeled as lower-case letters (a, b, c) and capital letters (A, B, C), respectively. Data is reported as mean ± SD (n=6).

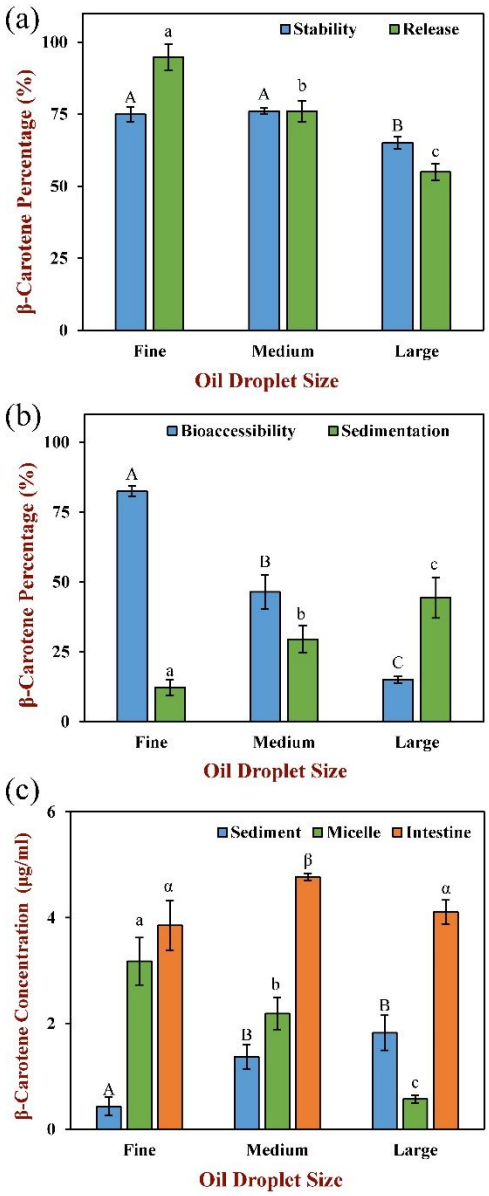


Fig. 7 The effect of oil droplet size on: (a) percentage of β-carotene that is stable and released; (b) percentage of β-carotene that is bioaccessible or sedimented; and (c) the concentration of β-carotene in different phases of corn oil-in-water emulsions after digestion in the small intestine. Capital letters (A, B, C), lower-case letters (a, b, c) and the Greek letters (α, β, γ) were used to designate significant difference ($p < 0.05$) among different oil droplet sizes. "Micelle" indicates the mixed micelle phase (supernatant fraction) of the intestinal samples, which contains micelles and vesicles. Data is reported as mean ± SD (n=6).

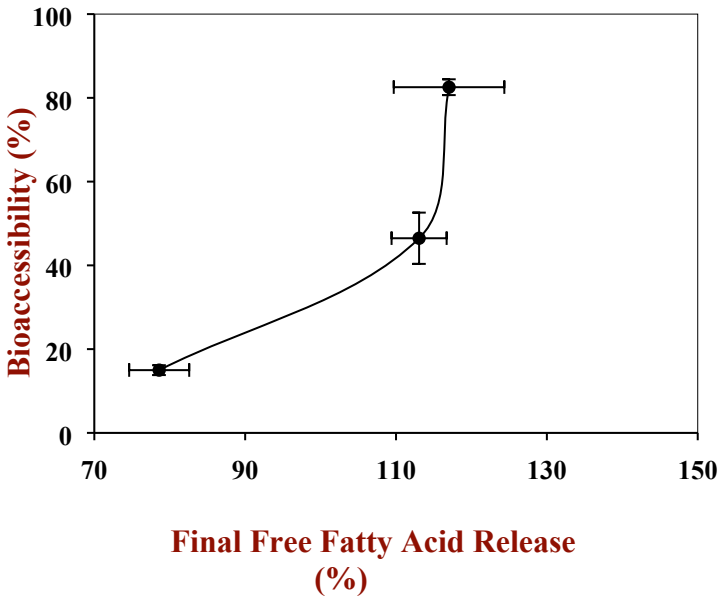
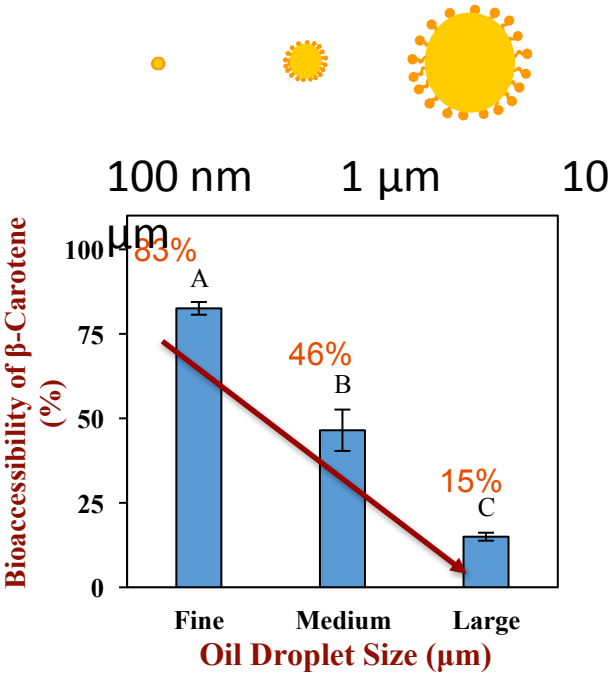


Fig. 8 Relationship between the final free fatty acids released and β-carotene bioaccessibility after *in vitro* digestion of emulsions with different droplet size. From left-to-right, the data correspond to large, medium, and fine emulsions. Data is reported as mean ± SD (n=6).

Highlights

- INFOGEST simulated gastrointestinal model used to study digestion and bioaccessibility.
- Emulsions with three different droplet sizes used: 0.16, 1.1 and 8.2 μm.
- The rate and extent of lipid droplet digestion increased with decreasing droplet size
- The bioaccessibility of beta-carotene increased with decreasing droplet size


100 nm
1 µm
10 µm
Bioaccessibility of β-Carotene (%)
100
75
50
25
0
83%
A
46%
B
15%
C
Fine
Medium
Large
Oil Droplet Size (µm)