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Factors impacting lipid digestion and β-carotene bioaccessibility assessed by standardized gastrointestinal model (INFOGEST): Oil droplet concentration

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Abstract

Food, nutrition, and pharmaceutical scientists are trying to elucidate the major factors impacting the bioavailability of macronutrients (*e.g.,* lipids) and micronutrients (*e.g.,* vitamins) so as to improve their efficacy. Currently, there is still a limited understanding of how food matrix effects impact digestion and bioaccessibility determined under the INFOGEST model, which is currently the most widely used standardized *in vitro* gastrointestinal model. Therefore, we examined the impact of corn oil concentration on lipid digestion and β-carotene bioaccessibility using model food emulsions. For all oil concentrations tested (2.5 to 20%), complete lipid digestion was achieved using fed-state gastrointestinal conditions, which could only be seen if a back-titration was performed. The particle size and negative surface potential on the mixed micelles formed at the end of the small intestine phase both increased with increasing oil concentration, which was attributed to the generation of more free fatty acids. The β-carotene bioaccessibility increased when the oil concentration was raised from 2.5 to 10% due to the increased solubilization capacity of the mixed micelles, but then it decreased when the oil concentration was raised further to 20% due to precipitation and sedimentation of some of the β-carotene. The maximum β-carotene bioaccessibility (93.2%) was measured at 10% oil. These results indicate that the oil concentration of emulsions influences β-carotene bioaccessibility by altering digestion, solubilization, and precipitation processes. This knowledge is important when designing more effective functional or medical food products.

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

1. Introduction

Historically, a number of commonly consumed food products have been fortified with essential micronutrients (vitamins and minerals) to prevent nutritional deficiencies in the general population, including milk, juice, cereal, and bread.[1, 2] More recently, there has been great interest in the fabrication and development of a new generation of fortified food products containing nutraceuticals.[3] These bioactive food components are claimed to exert beneficial health effects when consumed at sufficiently high levels over extended periods.[4] In particular, many nutraceuticals have been claimed to protect against chronic conditions such as cardiovascular disease, eye disease, brain disease, diabetes, hypertension, and cancer.[5] A growing number of consumers are therefore purchasing nutraceutical-fortified functional foods to improve their health and wellbeing, with the aim of increasing their lifespan and quality of life.[6, 7] In the future, it is hoped that functional foods may play an important role in reducing the incidences of chronic diseases, thereby reducing the need for pharmaceutical or surgical interventions.[8, 9] The efficacy of functional foods depends on the bioavailability of the nutraceuticals they contain, which depends on the composition and structure of the surrounding food matrix.[10, 11] As a result, there has been a major research effort to elucidate the key factors affecting nutraceutical bioavailability so that more efficacious functional food products can be designed.[12, 13]

Many nutraceuticals are strongly hydrophobic substances that are challenging to incorporate into functional foods because of their poor water-solubility and bioaccessibility.[10, 14] For this reason, hydrophobic nutraceuticals are typically loaded into colloidal particles that have hydrophobic interiors and hydrophilic exteriors before being incorporated into functional foods.[13, 15, 16] Oil-in-water emulsions are one of the most commonly used colloidal systems for

encapsulating and delivering hydrophobic nutraceuticals because they can be economically fabricated using existing homogenization technologies.[14, 17] Nevertheless, the composition and structure of these delivery systems, as well as the surrounding food matrix, must be carefully designed to ensure good nutraceutical bioaccessibility.[18, 19] Indeed, a variety of food matrix effects impact nutraceutical bioaccessibility. For instance, multivalent cations (such as calcium and magnesium) reduced carotenoid bioaccessibility, which was due to their ability to form insoluble soaps with long-chain fatty acids thereby reducing the number of mixed micelles present.[20] Whey proteins have been shown to either increase or decrease β-carotene bioaccessibility, depending on the extent of digestion.[21] The presence of plant-based oils and dietary fibers has also been shown to either increase or decrease the carotenoids bioaccessibility vegetables depending of the nature of the meal they are included in.[22] An improved understanding of the impact of food matrix effects on nutraceutical bioaccessibility may therefore contribute to the formulation of functional food products with higher and more consistent biological activities.[23]

The impact of food matrix effects on the bioaccessibility of nutraceuticals is usually explored using gastrointestinal tract (GIT) models.[24, 25] These models are designed to simulate the conditions present within the different parts of the human GIT (mouth, stomach, and small intestine), such as incubation times, mechanical actions, pH, mineral compositions, enzyme activities, and other factors.[25] These *in vitro* models cannot account for the dynamic complexity of the real human GIT, but they can provide valuable insights into the physicochemical phenomenon involved since samples can easily be collected and characterized.[26, 27] Moreover, they can be used to rapidly screen formulations with different compositions and structures, which cannot be easily achieved using *in vivo* testing methods, due to cost, time, and ethical reasons.

One of the most widely used simulated GIT models was developed by the INFOGEST international consortium.[25, 27] The conditions used in this model have been standardized so that researchers from different laboratories can compare their results under similar conditions. At present, however, there is still a relatively lacking understanding of how different food matrix effects impact the bioaccessibility of nutraceuticals determined using this new simulated GIT model.

In this article, we use the updated INFOGEST method to study the impact of oil concentration on the bioaccessibility of β-carotene encapsulated within a model food emulsion. This carotenoid has an extremely low water-solubility and poor bioaccessibility in its pure crystalline form, which makes it a good candidate for encapsulation.[12] In addition, it exhibits provitamin A activity and is a natural antioxidant, which means that enhancing its bioaccessibility may have health benefits.[28] Moreover, β-carotene is a strongly pigmented substance so that is concentration can easily be quantified using simple UV-visible spectroscopy methods. This carotenoid has also been used extensively in previous GIT studies, which has led to a good understanding of the major factors impacting its bioaccessibility.[14, 29, 30] As a result, β-carotene serves as a useful model hydrophobic nutraceutical for comparing the efficacy of different approaches for increasing nutraceutical bioaccessibility.

The bioaccessibility of carotenoids depends on several factors: liberation from the food matrix; solubilization within mixed micelles; and interaction with other food ingredients.[31] Typically, the lipid phase surrounding the carotenoids must be digested before they are released. The products generated from lipid digestion, namely monoacylglycerols (MGs) and free fatty acids (FFAs) interact with bile salts, phospholipids and other lipophilic components to form mixed micelles that incorporate the carotenoids within their hydrophobic interiors. These

carotenoid-loaded mixed micelles then travel through the gastrointestinal fluids and across the mucus layer before reaching the epithelium cells where they are absorbed. It should be noted, however, that some food components (such as chitosan) may interact with the mixed micelles and cause them to precipitate, thereby preventing the carotenoids from reaching the epithelium cells.[32]

In vitro studies have shown that oil concentration can impact the bioaccessibility of carotenoids encapsulated in or ingested with oil-in-water emulsions.[33] For instance, increasing the oil content of emulsions has been shown to enhance the bioaccessibility of carotenoids, which was due to the formation of a higher number of mixed micelles that could solubilize the carotenoids.[34] Nevertheless, only a limited range of fat contents has been examined from previous literatures. In practice, emulsion-based functional foods fortified with nutraceuticals, such as beverages, creams, sauces, and dressings may contain a wide range of fat contents. Consequently, it is important to understand how β-carotene behaves in the gastrointestinal tract when it is present within food matrices with different oil contents. For this reason, the goal of the current paper was to determine the impact of oil droplet concentration on β-carotene bioaccessibility in model food emulsions using the standardized INFOGEST method.[25] We hypothesized that the concentration of oil droplets initially present would alter the bioaccessibility of the carotenoids by altering lipid digestion, micelle solubilization, and/or micelle precipitation. The knowledge gained from this study should aid in the design of more effective functional food products.

2. Materials and methods

2.1. Materials

Corn oil (Mazola, ACH Food Companies, Memphis, TN, USA) was purchased from a supermarket. Tween 20 was purchased from ACROS Organic (Pittsburgh, PA, USA). Chemicals purchased from the Sigma-Aldrich Company (St. Louis, MO, USA) included β-carotene (synthetic, ≥93% in UV); porcine gastric mucin; pepsin from porcine gastric mucosa (≥250 units/mg); pancreatin from porcine pancreas; porcine lipase (100-400 units/mg); and, porcine bile extract. Information about the methods used to measure enzyme activity are included in the supplier's website. 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 (18 MOhm·cm) obtained from a water-purification system (Nanopure Infinity, Barnstaeas International, Dubuque, IA, USA).

2.2. Preparation of emulsion-based delivery systems

Carotenoid-fortified delivery systems were fabricated according to a method described previously.[35] An aqueous phase was prepared by dispersing non-ionic surfactant (2.0 wt% Tween 20) in phosphate buffer solution (5 mM, pH 7.0). The oil phase was prepared by dispersing β-carotene (0.1 wt%) in warmed corn oil (50 ºC) by repeated sonication (40 kHz, 1 min) and stirring (5 min) cycles until fully dissolved (clear solution). The oil phase (20 wt%) and aqueous phase (80 wt%) were combined together using a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) (10,000 rpm, 2 min), and then homogenized using a microfluidizer (M110Y, Microfluidics, Newton, MA) (12000 psi, 3 passes). Emulsions with

lower oil concentrations (2.5, 5 and 10%) were then prepared by dilution of the stock emulsion with phosphate buffer solution.

2.3. Measurement of particle size

The dimensions of relatively large particles (initial emulsions and digested emulsions) were determined using a static light scattering particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Before measurement, samples were diluted with buffer solution (same pH) and stirred (1200 rpm) to ensure they were homogeneous, the light scattering signal was high enough to obtain reliable results, and any multiple scattering effects were minimum. A phosphate buffer solution with an appropriate pH was used to dilute the samples: initial, oral and small intestine (pH 7); stomach (pH 3). Appropriate refractive indices were used for the oil phase (1.472) and aqueous phase (1.33) when converting the light scattering pattern into a particle size distribution. The results were then reported as the surface-weighted mean particle diameter ($D_{3,2}$) calculated from the full particle size distribution.

The dimensions of relatively small particles (mixed micelles) were determined using a dynamic light scattering particle size analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, Worcestershire, UK). The samples were diluted with phosphate buffer solution (pH 7) before analysis to obtain an appropriate signal intensity for reliable measurements. The same refractive indices were used in the analysis as for the static light scattering measurements. The results are reported as the Z-average diameter.

2.4. Surface potential characterization

The surface potential (ζ-potential) of the particles in the emulsions and digested emulsions was measured using a dedicated microelectrophoresis instrument (Zetasizer Nano ZS, Malvern

179 Instruments). Sample dilutions were performed using the same phosphate buffer solutions
180 described for the particle size measurements.

181 **2.5. Microstructural analysis**

182 Confocal microscopy analysis was performed according to a method described previously.[36]
183 Briefly, the oil phase was dyed with Nile red solution and then the resulting samples were
184 imaged using a confocal laser scanning microscope (Nikon D-Eclipse C1 80i, Nikon, Melville,
185 NY, USA).

186 **2.6. *In vitro* digestion**

187 *In vitro* digestion of the emulsion samples was performed using the recently updated
188 INFOGEST gastrointestinal tract simulation method [25], with some slight modifications. Initially,
189 enzyme characterization assays were performed to establish the optimum enzyme activities
190 required as described in the INFOGEST method. The temperature was maintained at 37 °C for
191 the whole digestion process, and preheated solutions were used throughout the procedure to
192 avoid any temperature fluctuations (which might impact enzyme activity).

193 **Oral phase**: Simulated saliva fluids containing 0.00375 g/ml mucin were mixed with
194 emulsion samples at a ratio of 1:1 w/w. The mechanical forces experienced by foods in the
195 human mouth were simulated using a mechanical shaking device (Model 4080, New Brunswick
196 Scientific, New Brunswick, NJ, USA) operating at a speed of 100 rpm. The samples were
197 maintained in the oral phase for 2 min.

198 **Gastric phase**: The sample exiting the oral phase was mixed 1:1 (v/v) with simulated
199 gastric fluids containing pepsin (2000 U/ml in the final digestion mixture), and then the system
200 was adjusted to pH 3.0 to trigger gastric digestion. The mechanical forces applied to the sample

were the same as those employed in the oral phase. The sample was maintained under simulated gastric conditions for 2 h.

Intestine phase: The sample exiting the gastric phase was further diluted 1:1 (v/v) with simulated small intestinal fluids containing pancreatic enzymes and 10 mM bile salts. Pancreatin and pancreatic lipase were added to reach a trypsin activity of 100 U/ml and a lipase activity of 2000 U/ml in the final mixture. An automatic titration device (857 Titrando, Metrohm USA Inc., Hillsborough, FL, USA) was used to maintain the sample at pH 7.0. The sample was maintained under simulated small intestinal conditions for 2 h. After this time, the intestinal sample was centrifuged (Sorvall Lynx 4000 centrifuge, Thermo Scientific, Waltham, MA, USA) at 46,285 ×g (18,000 rpm, 4 °C) for 50 min to separate the mixed micelle and sediment phases.

2.7. Measurement of lipid digestion

Lipid digestion was quantified by converting the volume of NaOH titrated into the reaction vessel into the fraction of free fatty acids released. The titration process was separated into two steps. In the first step, the volume of NaOH solution required to maintain the system at pH 7.0 throughout the small intestine phase was recorded. In the second step, the volume of NaOH solution required to titrate the solution to pH 9.0 was recorded. This second step is required because not all of the free fatty acids generated during lipid digestion are fully deprotonated at neutral pH. The total volume of NaOH from these two steps was then used to calculate the total amount of FFAs released. The effect of any non-lipid components (the “blank” test) that might have contributed to the measured volume was accounted for by subtracting the volume of NaOH required to titrate a sample containing no oil (but otherwise the same as the test sample). The mathematical calculation was performed according to a method described previously.[37, 38]

2.8. Extraction and analysis of β-carotene

The β-carotene in the digested samples was extracted and analyzed according to a method described in a previous study [39] with slight modifications. Briefly, an organic solvent consisting of 2:3 (v/v) hexane/isopropanol was used to carry out the extraction of the carotenoids. An aliquot of 0.5 ml sample was mixed with 1.2 ml of organic solvent. This mixture was then centrifuged (Minispin centrifuge, Eppendorf North America, Inc., Hauppauge, NY, USA) at 6000 rpm for 2 min, the supernatant was collected, and then its absorbance at 450 nm was measured using a UV-visible spectrophotometer (Genesys 150, Thermo Scientific, Waltham, MA, USA). 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).

2.9. Statistical analysis

The emulsion preparation was carried out in duplicate, and the digestion process and other characterization assays were carried out in triplicate. The means and standard deviations of these measurements were then calculated. The statistical differences among samples were calculated at

a confidence level of 95% using ANOVA with Tukey test. SPSS software (IBM Corp., Armonk, NY, USA) was used to perform all statistical calculations.

3. Results and discussion

3.1. Physical and structural properties of emulsions during digestion

Initially, the impact of oil droplet concentration (2.5, 5, 10 and 20%, w/w) on the gastrointestinal behavior of carotenoid-fortified emulsions was examined. These emulsions all had a fixed surfactant-to-oil ratio of 1:10 (w/w) and a fixed carotenoid-to-oil ratio of 1:1000 (w/w).

The surface weighted mean particle diameter ($D_{3,2}$) of the initial stock emulsion was 0.158 ± 0.001 μm, indicating that the combination of surfactant and homogenization conditions used in our study were efficient at creating small oil droplets. Tween 20 is a relatively hydrophilic non-ionic surfactant (HLB = 16.7) that can rapidly absorb to oil droplet surfaces and stabilize them against aggregation.[40] The surface potential of the Tween 20-coated oil droplets in the initial emulsion was -18.0 ± 0.8 mV. This relatively high negative charge may be ascribed from preferential adsorption of anionic hydroxyl ions (OH^-) from the water or from the presence of anionic impurities in the surfactant, such as free fatty acids.[41]

The *in vitro* gastrointestinal fate of the emulsions was established by passing them through the INFOGEST model.[25] Changes in the physical and structural properties of different emulsion samples were determined after each digestion step to provide some insights into the key factors imparting lipid digestion and carotenoid bioaccessibility.

After the oral phase, the $D_{3,2}$ values of all the samples remained relatively small, ranging from about 0.158 to 0.170 μm (Fig. 1a). This result suggests that the surfactant-coated oil droplets were relatively stable against aggregation within the oral phase. The ζ-potential of the

oil droplets was also fairly similar before (-18 mV) and after (-18 to -19 mV) exposure to the oral phase (Fig 1b), suggesting that there was not a major change in interfacial composition.[42] The presence of the non-ionic surfactant would be expected to inhibit the attachment of other substances to the droplet surfaces, such as mucin.

After the stomach phase, the $D_{3,2}$ values of all the emulsions were fairly similar to those obtained in the oral phase, ranging from around 0.159 to 0.196 μm (Fig. 1a), indicating that the oil droplets were relatively stable to aggregation in the simulated gastric solution conditions. However, the magnitude of the surface potential on the oil droplets decreased significantly ($p < 0.05$) after exposure to the stomach phase, reaching values between about -1.9 and -1.2 mV (Fig. 1b). Similar results have also been reported by other researchers monitoring the behavior of Tween 20-stabilized emulsions in a simulated gastric environment.[43] In other words, these results indicate that the surfactant-coated oil droplets were resistant to aggregation when exposed to the highly acidic conditions in the stomach,[44] presumably because they were primarily stabilized by steric repulsion between the hydrophilic polyoxyethylene head-groups of the surfactant molecules, rather than by electrostatic repulsion. The reduction in the negative charge on the droplet surfaces may have been due to protonation of any anionic impurities (such as free fatty acids) or due to a reduction in the adsorption of OH^- groups under acidic conditions.

A number of researchers have shown that the digestion of lipids within the small intestine depends on the aggregation state of the oil droplets exiting the stomach.[45-47] For this reason, the gastric samples were initially adjusted to pH 7 (without lipase and bile salt addition) to represent the small intestine phase before lipid digestion ("SI-Initial"). The size of the droplets remained relatively small when the condition was elevated to pH 7, again showing that they were stable in maintaining the oil droplet size under neutral conditions. Moreover, the magnitude of the ζ-

potential became strongly negative again, around -17.2 to -22.3 mV (Fig. 1b), suggesting that the oil droplet surfaces once again contained some anionic substances, such as fatty acids or hydroxyl ions. It should be noted that oil droplet concentration did not have a major impact on the size, surface charge and microscopic properties of the emulsions in the oral, gastric, or initial small intestine phases.

There was, however, a pronounced change in the properties of the oil droplets by the end of the small intestine phase, after bile salts and lipase was added. Moreover, the extent of this change depended on oil concentration. At the end of the small intestine digestion, the mean size value ($D_{3,2}$) increased with increasing oil concentration, from 0.273 μm for 2.5% oil to 0.549 μm for 20% oil (Fig. 1a). These observations corresponded with the confocal microscopy images, which also showed an increase in the size of colloidal lipid particles with higher oil concentration (Fig. 3a). Interestingly, the particle size (measured by dynamic light scattering) and turbidity (measured by UV-visible spectroscopy) of the mixed micelles produced by lipid digestion also increased with increasing oil concentration (Figs. 2 and 3b). In general, these samples may contain undigested oil droplets, micelles, vesicles, and/or insoluble calcium soaps, whose size could all change depending on the initial oil concentration in the emulsions. Simple micelles typically have diameters around 10 nm or less, but the vesicles and other components in the mixed micelle phase lead to much higher particle sizes [48].

β-carotene bioaccessibility would be expected to increase as the amount of mixed micelles formed by lipid digestion increased.[49] Consequently, one would expect the bioaccessibility to increase as the oil concentration increased. Conversely, the bioaccessibility would be expected to decrease as the amount of insoluble sediment from the samples increased. In this study, we

observed that the quantity of sediment formed increased as the oil concentration increased (Fig. 3c).

After the intestinal phase, the absolute value of the negative surface potential increased as the oil concentration increased, rising from -30.5 mV for 2.5% oil to -59.4 mV for 20% oil (Fig. 1b). This effect can be ascribed to the generation of more anionic fatty acids during lipid digestion, which were present at the surfaces of the colloidal particles in the digested samples. The ζ-potential of the mixed micelle phase also increased as the oil concentration increased, rising from -30.4 mV for 2.5% oil to -68.0 mV for 20% oil (Fig. 1b). Again, this effect can be explained by the presence of a higher level of anionic fatty acids after lipid digestion, which would be incorporated into the mixed micelles. The average size of the mixed micelles also increased with increasing oil concentration (Fig. 2). This suggests that there may have been larger micelles or vesicles formed at higher FFA levels, or that there was an increase in the vesicle-to-micelle ratio. At high oil concentrations, the fatty acids appeared to be incorporated into insoluble calcium soaps rather than into mixed micelles, based on the fact that more sediment was observed at the bottom of the samples (Fig 3c).

3.2. Lipid digestion in the intestinal digestion

The amount of FFAs released from the lipid phase in the different emulsions was monitored using the pH-sat method (Fig. 4). The FFAs released was measured using a two-step procedure: (i) at pH 7 during digestion, which only detects the ionized fatty acids formed under neutral conditions; (ii) by back-titrating to pH 9, which also detects any fatty acids that were not ionized under neutral conditions. At pH 7, the fraction of FFAs released during digestion decreased significantly ($p < 0.05$) with increasing oil concentration, changing from 79.0% for 2.5% oil to 47.7% for 20% oil (Fig. 4a). Thus, the percentage of fatty acids measured by the end of lipid

digestion was considerably less than 100%. Previous studies have shown that a fraction of the FFAs released during lipid digestion are not ionized at pH 7, so they are not titrated by NaOH during pH stat measurements.[38, 50] A back titration was therefore carried to measure these non-ionized fatty acids.

After titration to pH 9, the percentage of FFAs produced still tended to decrease with increasing oil concentration (Fig. 4a). Interestingly, though, the amount of FFAs produced was considerably higher than 100% (assuming two fatty acids formed per triacylglycerol). For instance, the final amount of FFAs released was calculated to be around 127% for the emulsions initially containing 2.5% oil. The reason for the higher amount of lipid digestion than expected may be that some of the monoacylglycerols were converted into a glycerol molecule and a fatty acid under alkaline conditions.[51] This phenomenon appears to be an important limitation of the INFOGEST method for monitoring lipid digestion.[51] Overall, our results suggest that the majority of the fat droplets was digested in all systems.

It should be noted that the dependence of the final FFAs released on oil concentration was different at pH 7 and pH 9. This suggests that the ratio of ionized-to-non-ionized FFAs in the digested emulsions depended on their initial oil concentration. For this reason, the ratio of final FFA levels at pH 7 to pH 9 was calculated: 0.62, 0.51, 0.46 and 0.48 at 2.5, 5, 10, and 20% oil, respectively. Thus, when there is more oil phase present the FFAs have a greater tendency to be in the non-ionized state. Previous studies have reported that the pK_a values of fatty acids increases as their concentration increases, especially for long-chain ones.[52] Indeed, the pK_a values of long-chain fatty acids have been shown to be highly dependent on their local environment.[51] This phenomenon was mainly attributed to interactions between the polar head-groups of fatty acids when they are in close proximity.

The generation of FFAs during the course of lipid digestion was calculated using the measured NaOH volumes and a correction factor: CF = Final FFAs(pH 9)/Final FFAs(pH 7) (Fig. 4b). In addition, the molar concentration of FFAs produced during digestion was calculated (Fig. 4c). For all oil concentrations, the amount of FFAs generated increased steeply during the first 500 s and then more gradually afterward. These results suggest that the lipase rapidly adsorbed to the surfaces of the oil droplets and initiated digestion of the underlying triacylglycerols.[53] At the end of the small intestine period, there appeared to be a fairly similar total amount of lipid digestion for the emulsions containing 2.5 to 10% oil, but a reduced amount for the emulsions containing 20% oil (Fig. 4b). This result suggests that some of the lipids may not have been digested at the highest oil concentrations used, which may have been due to the limited amount of lipase, bile salts, and calcium present in the *in vitro* GIT model. Conversely, the absolute amount of FFAs produced during lipid digestion increased as the oil concentration increased (Fig 4c), which should be expected because there were more triacylglycerol molecules present to convert into fatty acids.

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

After passing through the full INFOGEST digestion method, the β-carotene concentration was measured in the mixed micelle phase, the sediment phase, the total digest and the initial emulsions. These values were then used to determine the stability, release, and bioaccessibility of the carotenoid.

Stability: The stability of the carotenoids was calculated by comparing the total β-carotene concentration from the small intestine samples with that in the original emulsion, accounting for the dilution steps (Fig. 5a). Carotenoid stability was fairly similar for all oil concentrations used (2.5 to 20%), ranging from around 70 to 80%. These values agree with those published

previously for related systems.[54] We postulate that the observed reduction in total β-carotene concentration by the end of the INFOGEST model was mainly due to chemical degradation of the carotenoids. The highly acidic gastric fluids within the simulated stomach, as well as the slightly elevated temperature that the samples were exposed to during the whole digestion (37 ^{o}C), would have accelerated any acid-induced chemical degradation of the carotenoids.[55] Moreover, the samples were exposed to light for short periods during the experimental operation (*e.g.,* when transferring samples between digestion phases), which may also have accelerated the chemical degradation of the carotenoids. In addition, some of the carotenoids may also have adhered to the surfaces of the containers or other apparatus used in the simulated digestion method, which would also have reduced their concentration in the small intestine phase. Overall, our results suggest that the oil droplet concentration did not strongly impact the chemical stability of the β-carotene in the emulsions. This may have been because the initial concentration of the carotenoids in the oil droplets (0.1%) was the same in all systems.

Release: The release of the carotenoids was calculated as the sum of β-carotene in the micelle and sediment phases divided by the total β-carotene in the digest. The release rate was only around 80.5% for the emulsions containing 2.5% oil, but close to 100% for all the other emulsions (Fig. 5b). This result suggests that it is harder to efficiently extract the carotenoids from the samples when the initial oil concentration (and therefore final mixed micelle concentration) is relatively low. Thus, in future studies on food matrix effects, a higher oil concentration is recommended.

Bioaccessibility: The bioaccessibility of the carotenoid was the fraction of β-carotene from the total digest that was dissolved in the mixed micelle phase. Similarly, the fraction of carotenoids in the sediment was calculated by comparing the concentration of β-carotene in the

403 sediment with that in the total digest. As the oil concentration increased from 2.5 to 10%, the
404 bioaccessibility of carotenoids in the mixed micelle phase increased from 60.5% to 93.2%,
405 whereas that in the sediment phase decreased from 20.4 to 7.9% (Fig. 5b). The initial increase in
406 bioaccessibility with increasing oil concentration can be attributed to the generation of more
407 mixed micelles available of solubilizing the carotenoids. When reported as an absolute β-
408 carotene concentration, the quantity of carotenoids in the mixed micelle phase increased from
409 1.20 to 6.77 μg/ml when the oil concentration was raised from 2.5% to 10%, while the total
410 quantity of carotenoids in the small intestine phase increased from 2.02 to 7.24 μg/ml,
411 respectively (Fig. 5c). This result highlights the fact that it is necessary to use a higher oil
412 concentration to increase the total amount of carotenoids available for absorption in the small
413 intestine. As expected, the β-carotene concentration in the sediment phase slightly increased
414 when the oil concentration was raised being 0.357, 0.432, and 0.543 μg/ml for 2.5, 5.0, and 10%
415 oil, respectively (Fig. 5c). The β-carotene in the sediment phase is probably trapped within
416 insoluble calcium soaps formed by bile salts, free fatty acids and calcium ions.
417 Interestingly, increasing the oil concentration in the emulsions from 10 to 20% led to an
418 inhibition in β-carotene bioaccessibility from 93.2% to 80.3% (Fig. 5b). This effect can be due to
419 an increase in the quantity of carotenoids within the sediment phase at the highest oil
420 concentration, *i.e.,* 1.20 μg/ml or 14.3% (Figs. 5b and 5c). We hypothesize that this effect is due
421 to the formation of a greater amount of sediment at higher oil concentrations, as seen by visual
422 appearance of the samples (Fig. 3c). The origin of this effect may be due to the increase in the
423 pK_a values of the long-chain FFAs at higher oil concentrations.[52] As a result, a lower fraction of
424 these fatty acids would be ionized at neutral pH, making them less water-soluble and more prone
425 to forming insoluble sediments. Consequently, some of the carotenoids would be trapped within

these sediments. Researchers have suggested that hydrophobic nutraceuticals trapped within fatty acid-rich sediments are unavailable for absorption and so end up within the feces.[56] These results suggest that it is important to design functional foods that can prevent carotenoids being trapped within the insoluble precipitates formed from fatty acids during lipid digestion.

In a previous study on closely related systems, it was reported that the bioaccessibility of β-carotene decreased from around 84% to 39% when the oil droplet concentration was increased from 4% to 20% [33], which was a much bigger reduction than observed in the current study. Indeed, the carotenoid bioaccessibility only decreased from around 82.5% to 80.3% when the oil concentration was increased from 5% to 20% oil in our study. This large difference in results can be attributed to the fact that different *in vitro* digestion methods were used. In the previous study, the lipid droplets were not completely digested exiting the small intestine phase because the lipase concentrations used were much lower than those employed in the INFOGEST method. As a result, the β-carotene was not fully released from the lipid phase, as well as there were less mixed micelles available to solubilize it.

4. Conclusions

In this study, the impact of oil concentration (2.5 to 20%) on the bioaccessibility of β-carotene vehiculated within model food emulsions was investigated using the updated standardized INFOGEST gastrointestinal simulation. At the beginning of the small intestine digestion, the size and surface potential of the oil droplets in the emulsions was independent of oil concentration. At the end of the small intestine phase, almost complete lipid digestion was observed in all of the emulsions after a back titration was carried out. Even so, the measured degree of lipid digestion decreased with increasing oil concentration. Interestingly, the fraction of the fatty acids released was much higher than expected in some of the samples (>120%),

449 which was attributed to conversion of some of the monoacylglycerols into fatty acids and
450 glycerol, which is obviously a limitation of the INFOGEST method. The bioaccessibility of the
451 β-carotene increased from 60.5% to 93.2% when the oil concentration was raised from 2.5% to
452 10 %, but then decreased to 80.3% when the oil concentration was further raised to 20%. This
453 effect was ascribed from the precipitation of some of the long-chain fatty acids at higher oil
454 concentrations, which caused some of the carotenoids to be trapped inside the sediment phase.
455 These results suggest that the oil concentration in functional foods should be optimized to obtain
456 the highest bioaccessibility. On the other hand, the total amount of carotenoids actually available
457 for absorption does increase with increasing oil concentration, which means that it may be
458 necessary to include a certain amount of oil to reach a desired target dose of the carotenoids.
459

Conflicts of interest

There are no conflicts to declare.

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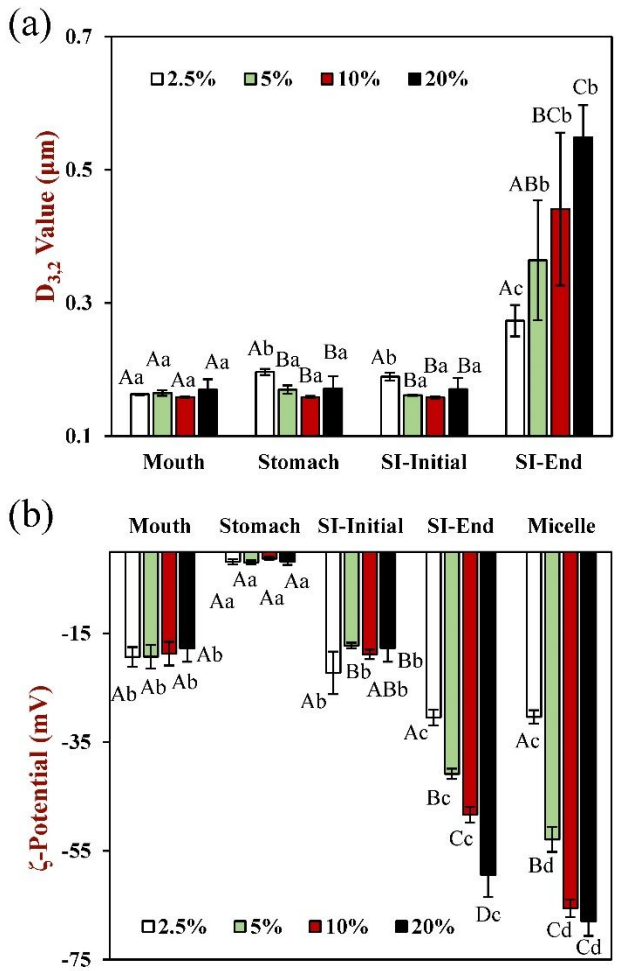


Fig. 1 The effect of different oil concentration on (a) the surface weighted mean particle diameter ($D_{3,2}$) measured by static light scattering and (b) ζ-potential measured by electrophoresis of the corn oil in water emulsion during *in vitro* gastrointestinal digestion. Different capital letters (A, B, C) were used to designate significant difference ($p < 0.05$) among oil concentration (same stage), and lower-case letter (a, b, c) for different stage (same oil concentration). SI is abbreviated for small intestine. Data is reported as mean ± SD (n=6).

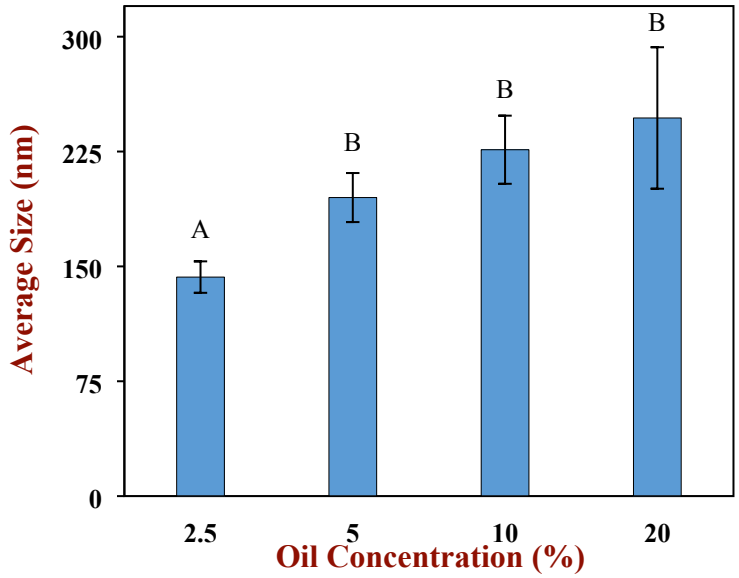


Fig.2 The average diameter of the particles in mixed micelle samples obtained after intestinal digestion of emulsions with different oil concentration. These measurements were carried out using dynamic light scattering. Capital letters (A, B, C) were used to indicate significant difference ($p < 0.05$) among samples. Data is reported as mean ± SD (n=6).

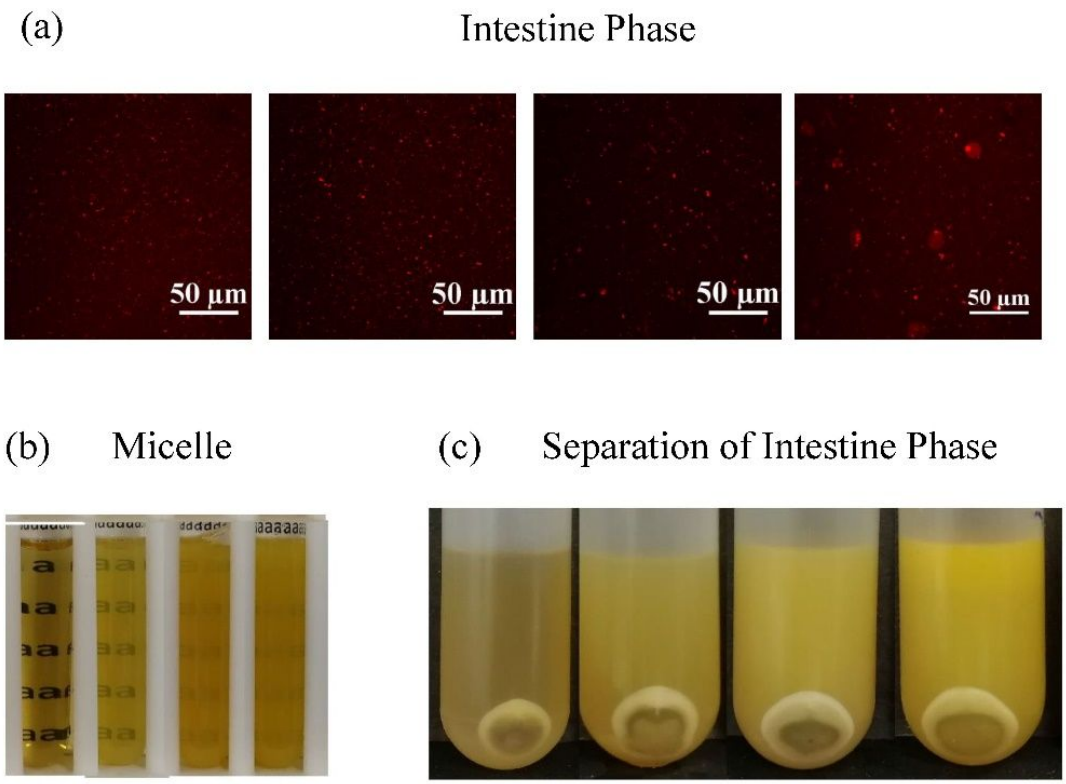


Fig. 3 The effect of different oil concentration on the (a) confocal photos of intestine samples, (b) optical photos of mixed micelle samples, and (c) the appearance of centrifugation separation of the intestinal samples (note the sediment at the bottom of the tubes). From left to right, the data correspond to the emulsions of increasing oil concentration from 2.5% to 20%.

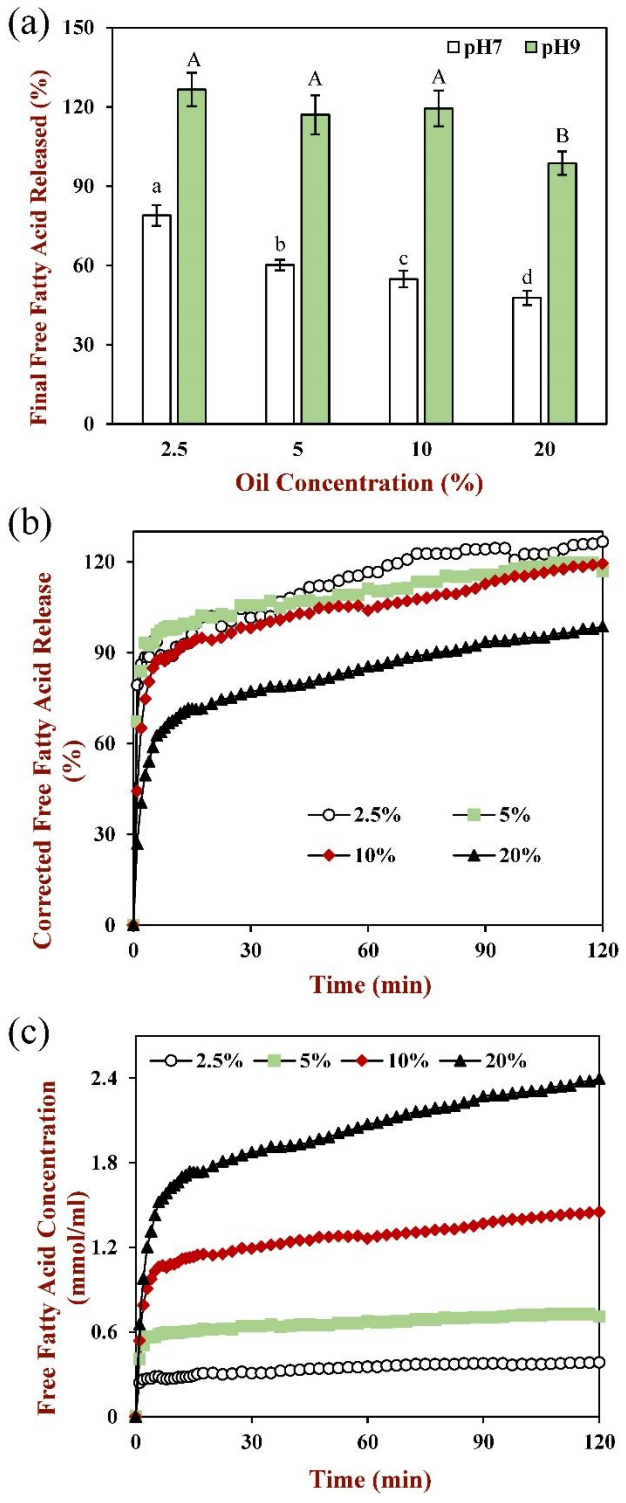


Fig. 4 The effect of different oil concentration on (a) final free fatty acid (FFA) released, (b) corrected FFA released profile, and (c) corrected FFA concentration profile of the corn oil in water emulsion during intestinal digestion. The significant difference among different oil

concentration for 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).

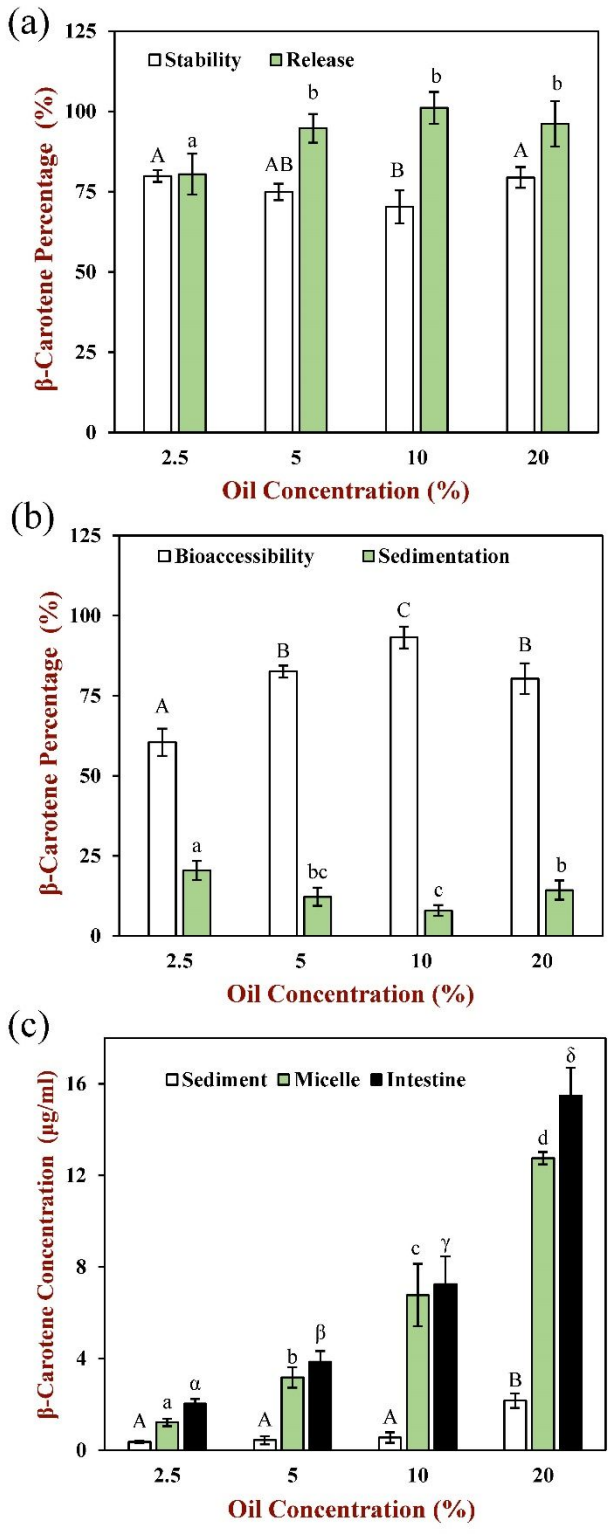
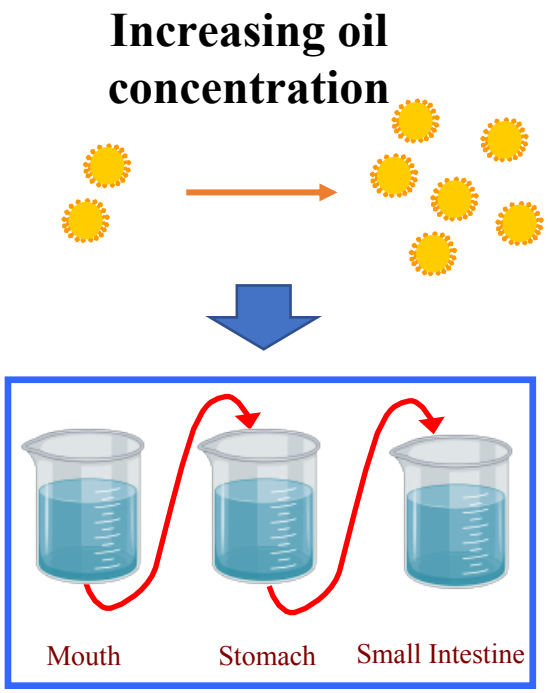


Fig. 5 The effect of different oil concentration on (a) stability and release, (b) bioaccessibility and sedimentation, and (c) β-carotene concentration in each phase of the corn oil in water emulsion after intestinal digestion. Capital letters (A, B, C), lower-case letters (a, b, c) and the

Greek letters (α, β, γ) were used to designate significant difference among different oil concentration. “Intestine” indicated the total digest obtained after intestinal digestion. Data is reported as mean ± SD (n=6).



INFOGEST *In vitro* Digestion