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Bioaccessibility of oil-soluble vitamins (A, D, E) in plant-based emulsions: Impact of oil droplet size

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Abstract

We systematically investigated the impact of oil droplet diameter (≈ 0.15, 1.6, and 11 μm) on the bioaccessibility of three oil-soluble vitamins (vitamin A palmitate, vitamin D, and vitamin E acetate) encapsulated within soybean oil-in-water emulsions stabilized by quillaja saponin. Lipid digestion kinetics decreased with increasing droplet size due to the reduction in oil-water interfacial area. Vitamin bioaccessibility decreased with increasing droplet size from 0.15 to 11 μm: 87 to 39% for vitamin A; 76 to 44% for vitamin D; 77 to 21% for vitamin E. Vitamin bioaccessibility also decreased as their hydrophobicity and molecular weight increased, probably because their tendency to remain inside the oil droplets and/or be poorly solubilized by the mixed micelles increased. Hydrolysis of the esterified vitamins also occurred under gastrointestinal conditions: vitamin A palmitate (~90%) and vitamin E acetate (~3%). Consequently, the composition and structure of emulsion-based delivery systems should be carefully designed when creating vitamin-fortified functional food products.

Keywords: droplet size; vitamin type; nanoemulsion; bioaccessibility; INFOGEST method.

1. Introduction

Vitamins are a group of organic molecules that play a critical role in many physiological functions important to human health. Deficiencies in these micronutrients leads to severe diseases. For example, lack of vitamin A leads to night blindness, lack of vitamin D leads to bone fractures and rickets, and lack of vitamin E leads to anemia and stunted growth. In addition to their role as micronutrients, oil-soluble vitamins may also have other therapeutic effects *e.g.,* by reducing the incidences of cancer, cardiovascular disease, and other chronic conditions [1]. Despite their beneficial health effects, many people do not get sufficient quantities of oil-soluble vitamins from their diet. Moreover, many individuals suffer from conditions that reduce the level of these vitamins absorbed from their foods, including the elderly and patients suffering from certain gastrointestinal conditions, or they have greater micronutrient demands than the general population, including babies, children, adolescents, pregnant women, and athletes [2].

Several strategies have been developed to tackle vitamin deficiencies or insufficiency, including food fortification and vitamin supplementation. These strategies have been shown to be effective at maintaining adequate vitamin levels within the human body, as well as at preventing or curing chronic diseases [3, 4]. For this reason, many food and supplement companies have developed products containing oil-soluble vitamins, which are designed to reduce deficiencies and/or improve health [5]. For example, milk is usually fortified with vitamin D, while many plant-based milk analogs are fortified with vitamins A, D, and E. Oil-soluble vitamins cannot simply be mixed with fluid beverages because they are immiscible with water and would therefore separate. For this reason, they are usually mixed with an oil phase first, which is then homogenized to form vitamin-loaded lipid droplets that can then be dispersed within an aqueous environment [6].

In general, the nutritional impact of ingested oil-soluble vitamins depends on the amount present within the human body in a bioactive form [7]. This amount depends on the bioavailability of the vitamins, which depends on their bioaccessibility, transformation, and absorption within the gastrointestinal tract (GIT) [8]. An understanding of the physicochemical basis of the bioavailability of oil-soluble vitamins is critical for improving the nutritional impact of fortified foods. In general, vitamin bioavailability is governed by the gastrointestinal conditions within the individual consuming the food, as well as by the precise nature of the food consumed [9]. As an example, the bioavailability of vitamin E has been reported to vary from around 10% to 80% for different food matrices [10]. Similar food matrix effects have also been reported for the bioavailability of vitamins A and D [11, 12]. These results illustrate the importance of carefully designing the composition and structure of fortified foods so as to increase the bioavailability of encapsulated vitamins [9, 13].

The size of the oil droplets in emulsified foods can easily be varied by modification of the homogenization conditions or emulsifier properties [7]. Droplet size greatly influences the physicochemical properties, stability, sensory attributes, and bioavailability of vitamin-fortified emulsions. For instance, many *in vitro* studies have shown that increasing oil droplet size significantly reduces the rate and degree of lipid digestion [12, 14]. At the same fat content, reducing the droplet size increases the droplet surface area, thereby promoting lipase adsorption and increasing lipolysis. However, the nature of these effects depends on emulsifier type, as this can impact the stability of the lipid droplets to flocculation or coalescence within the mouth, stomach, and small intestine [15]. As a result, the size of the lipid droplets within the small intestine may be very different from those in the ingested emulsions. It should be noted, producing emulsions with small droplets is typically expensive because specialized homogenization equipment is required, such as microfluidizers, high-pressure valve homogenizers, or sonicators [16]. Consequently, it is vital to optimize the droplet size for specific applications. For oil-soluble vitamins, the impact of droplet size on vitamin bioavailability is particularly important. Previous publications have shown that the bioaccessibility of encapsulated pro-vitamin A (β-carotene) increases as the oil droplet size decreases, which was attributed to faster and more extensive lipid digestion and mixed micelle formation [17]. Moreover, a recent study showed there was a reduction in the level of crystalline β-carotene present within the sediment phase formed after lipid digestion as the oil droplet size decreased, which was also attributed to faster and more extensive mixed micelle formation [18]. These results indicate that oil droplet size plays a critical role in determining the bioaccessibility of hydrophobic bioactives.

There is also strong evidence that the bioaccessibility of hydrophobic bioactives depends on their molecular dimensions relative to the dimensions of the hydrophobic regions inside mixed micelles. In particular, hydrophobic bioactives must be small enough to be accommodated within the hydrophobic domains of the mixed micelles. For example, small bioactives can easily be incorporated into mixed micelles formed from fatty acids with chain lengths varying from medium to long, thereby leading to a relatively high bioaccessibility [19]. Conversely, large bioactives can only be incorporated into mixed micelles formed from long chain fatty acids, otherwise they tend to precipitate, leading to a relatively low bioaccessibility [20]. The bioaccessibility also depends on the hydrophobicity of the bioactives: the transfer of a bioactive substance from the oil droplets to the mixed micelles tends to decrease as its hydrophobicity increases, thereby reducing the bioaccessibility [12, 21]. These results indicate that the molecular characteristics of hydrophobic bioactives also play a major role in determining their bioaccessibility.

In this study, we focus on the impact of oil droplet size on the gastrointestinal fate of three important oil soluble vitamins (vitamins A, D, E) encapsulated within model plant-based food emulsions. We focused on this type of emulsion because there has been growing interest in the utilization of plant-based foods within the food industry due to their perceived environmental, health, and ethical benefits. Thus, it is important to understand the factors that impact the bioavailability of micronutrients delivered in this new generation of nutritionally-fortified plant-based foods. A standardized *in vitro* digestion model (INFOGEST) was used to evaluate the hydrolysis and bioaccessibility of the vitamins and lipid phase, as well as the physical and structural changes in the emulsions during gastrointestinal tract (GIT) passage. A major focus of this study was to provide insights into the physicochemical mechanisms underlying the effects of oil droplet size and vitamin type on the bioaccessibility of oil-soluble vitamins in plant-based emulsions. We hypothesized that the bioaccessibility of the vitamins would increase with decreasing droplet size, but by an amount that depended on their molecular characteristics. In particular, we hypothesized that the release of the vitamins from the oil droplets would decrease as their hydrophobicity increased, whereas their solubilization in the mixed micelles would decrease as their molecular dimensions increased. Vitamins A, D, and E are widely used in commercial fortified foods and supplements and so the results of this study have practical relevance in the design for these products. For this reason, we used retinyl palmitate and tocopheryl acetate versions of vitamin A and E, respectively, since these esterified forms are widely used to prevent their oxidation during storage, thereby increasing their bioactivity [10, 22]. The results of this study should advance the understanding of food matrix effects on vitamin bioavailability, which may lead to the development of more effective functional foods and supplements.

2. Materials and methods

2.1 Materials

Soybean oil (Wesson, Conagra Brands, Inc., Chicago, IL, USA) was purchased from a local supermarket. Quillaja saponin (Q-Naturale 200 V) was kindly provided by Ingredion Inc. (Westchester, IL, USA). Vitamin A palmitate (1.7 Mio IU/G, stabilized with tocopherol), vitamin D_3 (1.0 Mio IU/G) and vitamin E acetate (98%) were kindly supplied by BASF corporation (Ludwigshafen, Germany). The reagents needed for the INFOGEST *in vitro* digestion experiments were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), including porcine gastric mucin, pepsin from porcine gastric mucosa (250 units/mg), pancreatin from porcine pancreas, porcine lipase (100-400 units/mg), porcine bile extract, and bile acid assay kit. All other chemicals and reagents were analytical grade or higher. The double distilled water for solution preparation was produced by a water-purification system (Nanopure Infinity, Barnstaeas International, Dubuque, IA, USA).

2.2 Emulsion preparation

The method used to prepare the emulsions was similar to that used in our previous publication, with some slight modifications [18]. Briefly, emulsions were prepared by homogenizing an oil phase (5 wt.%) into an aqueous phase (95 wt.%) containing emulsifier. In this study, two sets of oil phases were used for different research purposes. First, pure oil phase (soy oil) was used for the analysis of oil droplet size on the physicochemical properties, structure, and digestion of the emulsion samples within the simulated GIT. Second, vitamin-loaded oil phase was used to study the bioaccessibility and transformation of the vitamins in the simulated GIT. The vitamin amount used was 10-times higher than the recommended daily allowance (RDA) for each type of oil-soluble vitamin. Information about the properties of the different vitamins is included in Table 1 and Fig 1. The aqueous phase was the same for all the emulsions, and was prepared by dissolving 0.5 wt.% of quillaja saponin (based on the final emulsion) in phosphate buffer solution (5 mM, pH 7.0).

Emulsions with different oil droplet sizes were prepared by using different homogenization approaches. Emulsions containing relatively large oil droplets (around 10 μm: "large" emulsions) were prepared using a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) operated at 10,000 rpm for 4 min. Emulsions containing medium oil droplets (around 1 μm: "medium" emulsions) were prepared by further homogenization of the large emulsions using sonication (Sonicator FB505, Thermo Fisher Scientific, Waltham, MA, USA). The sonication conditions used were as follows: frequency = 20 kHz, amplitude = 20%, sonication on/off duration = 2/2 s, total sonication time = 3.5 min. Emulsions containing fine droplets (around 0.1 μm: "fine" emulsions) were prepared by microfluidization (M110Y, Microfluidics, Newton, MA) of the large emulsions at 12,000 psi for 3 circulations.

Table 1. Molecular and physicochemical characteristics of the oil-soluble vitamins[1] used in this study, as well as information about the amount of vitamin in the oil phase. *Key:* RDA = recommended daily allowance.

	Molar mass (g/mol)	**Log K_{OW}**	**RDA[3]**	**Vitamin in oil phase[2] (wt. %)**
Vitamin A-palmitate	524.9	14.8	800 μg/d	0.63
Vitamin A (retinol)	286.5	5.7	-	-
Vitamin D	384.6	7.5	15 μg/d	2.4
Vitamin E-acetate	472.7	10.9	15 mg/d	2.43
Vitamin E (α-tocopherol)	430.7	10.7	-	-

[1] The data were obtained from National Center for Biotechnology Information, U.S. National Library of Medicine, and ChemSpider, Royal Society of Chemistry.

[2] The vitamins were added at 10-times of the RDA in this study.

[3] Average of recommended daily allowances for male and female.

2.3 *In vitro* digestion

The *in vitro* digestion experiment was performed according to the standardized INFOGEST method [23], with some slight modifications [24]. Briefly, the *in vitro* digestion model included oral, gastric, and intestinal phases. In each phase, the sample was mixed with the digestive fluids at a volume ratio of 1:1, and then placed in an incubation device at 37 °C for a certain period of time. Specifically, in the oral phase, the original emulsion samples were mixed with simulated saliva solutions containing mucin (0.00375 g/ml) for 2 min. Afterwards, the oral samples were mixed with simulated gastric solutions containing pepsin (2000 U/ml in the final digestion mixture), the pH was adjusted to 3, and then the incubation lasted for 2 h in the gastric phase. In the oral and gastric phases, the samples were incubated within a mechanical shaking device (Model 4080, New Brunswick Scientific, New Brunswick, NJ, USA) operated at a speed of 100 rpm. In the intestinal phase, the gastric chyme was mixed with simulated small intestinal solutions containing pancreatic enzymes and bile salts (10 mM). The pancreatic enzymes consisted of both pancreatin compound enzymes and extra pancreatic lipase to obtain 100 U/ml trypsin activity as well as 2000 U/ml lipase activity in the final mixture. The pH environment of the samples was maintained at 7 by an automatic titration device (857 Titrando, Metrohm USA Inc., Hillsborough, FL, USA), and the titrant volume was recorded. The small-intestine phase lasted for 2 h. The centrifugation (Sorvall Lynx 4000 centrifuge, Thermo Scientific, Waltham, MA, USA) was used to separate the micelle and sediment phases from the digested intestinal samples, and the conditions were 18,000 rpm, 4 °C for 50 min. The fraction of sediment formed was then calculated:

$$\text{Sedimentation} = 100\ \times \frac{W_{sediment}}{W_{intestine}}$$

Here $W_{intestine}$ and $W_{sediment}$ are the weights of the whole intestinal sample and of the sediment collected after centrifugation, respectively.

2.4 Measurement of lipid digestion

The lipid digestion kinetics within the intestinal phase was quantified by titration of the free fatty acids (FFAs) released from the hydrolyzed triacylglycerols with NaOH solution. In addition, a back titration step (pH 9) was applied after the small-intestine phase to calculate the amount of FFAs that were non-ionized at pH 7 [25]. Blank samples were also analysed (same composition, but no oil) to remove the impact of non-oil components on the titration. The fraction of FFAs released was calculated according to the method described previously [25, 26].

2.5 Average size, charge, and microstructure characterization

The size, charge and microstructure of the particles within the emulsions were analyzed according to the analytical methods described in a previous publication [18], with some slight modifications. Briefly, the size of relatively large particles in the initial emulsions and digested samples were analyzed by the static light scattering method (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK), whereas the size of the relatively small particles in the mixed micelle samples were analyzed by the dynamic light scattering method (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, Worcestershire, UK). The ζ-potential of all samples was determined by microelectrophoresis (Zetasizer Nano ZS, Malvern Instruments). Before size and ζ-potential measurements, all samples were diluted with phosphate buffer solutions with pH values corresponding to the sample: initial and oral (pH 6), stomach (pH 3), and small-intestine (pH 7). The refractive index values used in the calculations of particle size and ζ-potential were 1.475 for the oil phase and 1.33 for the aqueous phase, respectively.

For the confocal microscopy measurements, the samples were dyed by Nile red solutions at a ratio of 1:20 v/v, placed on microscope slides, and then imaged (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, USA) at excitation and emission wavelengths of 543 and 605 nm, respectively.

2.6 Extraction and measurement of vitamin A, D, E

The same solvent extraction protocol was used for all the oil-soluble vitamins. A 2 ml aliquot of sample was mixed vigorously with 2 ml of an organic solvent containing hexane and ethanol (1:1, v/v). The resulting mixture was then centrifuged (Sorvall ST 8 centrifuge, Thermo Scientific, Waltham, MA, USA) at 4000 rpm for 2 min to separate the organic phase from the aqueous phase. The upper organic phase was then collected. This procedure was carried out three times and the organic solvents were combined. A saturated sodium chloride solution was added to the extracted solvents and the mixture was centrifuged again to remove any remaining aqueous fractions. The organic supernatant was then collected and dried using nitrogen gas. The dried vitamins were dissolved in HPLC grade methanol and then passed through a 0.45 μm filter (VWR International, Philadelphia, PA, USA) to remove any particulate material prior to HPLC analysis.

Vitamin quantification was carried out using a reverse phase HPLC system (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA) equipped by a Zorbax SB-C18 column (4.6 ×250 mm, 5 μm, Agilent Technologies, Santa Clara, CA, USA). The operating conditions tailored for each vitamin type. Specifically, the mobile phase for vitamin A palmitate and vitamin A was pure methanol solution at 40 °C, and the detection wavelength used was 325 nm [27]. For vitamin D, a mixture of methanol:water (95:5, v/v) was used as the mobile phase at 25 °C for vitamin D, and the detection wavelength used was 265 nm [28]. For vitamin E acetate and vitamin E, a mixture of methanol and water (97:3 v/v) was used as the mobile phase at 30 °C, and the detection wavelength used was 286 nm. These conditions were based on a previous publication [29], with slight modifications. The flow rate (1ml/min) and injection volume (20 μL) were the same for all samples. Data analysis was carried out using the instrument software (Agilent ChemStation).

Data analysis was performed using a method published previously [24]. The transformation of the esterified vitamin A and E to the non-esterified forms was calculated using the following equation:

$$\text{Transformation} = 100 \times \frac{C_{vitamin}}{C_{vitamin} + C_{vitamin-ester}}$$

Here C_{vitamin} and $C_{\text{vitamin-ester}}$ are the molar concentrations of the pure vitamin and esterified vitamin in the overall digesta after simulated intestinal digestion, respectively.

2.7 Statistical analysis

Emulsion preparation was duplicated, and the digestion process and all measurements were triplicated. The results are presented as the mean and standard deviation after combining data from replicated measurements. Statistical differences for pairwise comparison were calculated at a confidence level of 95% using ANOVA with either a Duncan test (homogenous) or Dunnett's T3 test (inhomogeneous). All statistical calculations were carried out using SPSS software (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1 Physical and structural characterization during simulated GIT digestion

Oil-in-water emulsions with a wide range of target mean droplet diameters were prepared using different homogenization approaches: fine (≈ 0.1 μm), medium (≈ 1 μm) and coarse (10 μm) emulsions. The measured $D_{3,2}$ values of these emulsions were 0.149, 1.57, and 11.1 μm for microfluidization, sonication, and simple blending, respectively (Fig. 2a). These light scattering measurements were supported by the confocal microscopy images of the initial emulsions (Fig. 3), which showed that the size of the individual oil droplets increased from fine to medium to large emulsions. In all the initial emulsions, the oil droplets were evenly dispersed throughout the microscopy images. Microscopy analysis also indicated that there was a wide distribution of droplet sizes within each type of emulsion, particularly the coarse one. Visibly, a cream layer was formed on top of the large emulsions after they were stored for a few hours, which can be ascribed to the rapid upward movement of the large droplets due to gravity (data not shown). In contrast, the fine and medium emulsions remained homogeneous after a few hours of storage because the smaller droplets they contained creamed more slowly. These results suggest that vitamin-fortified emulsions should contain relatively small oil droplets (≤1 μm) if they are supposed to remain physically stable during storage. However, this problem may be overcome if they have a high viscosity or are gelled.

The particles in the initial emulsions were all negatively charged, with the ζ-potential values ranging from around -68.4 to -57.0 mV (Fig. 2b), which can be attributed to the fact that quillaja saponin contains anionic carboxyl groups above pH 3 [30]. There was a significant ($p < 0.05$) difference in the ζ-potential of emulsions containing different oil droplet sizes, which may be ascribed to either incomplete coverage of the saponin on the interface (fine emulsions) or the presence of non-adsorbed saponin molecules (large emulsions) [31]. In addition, sonication might have promoted the formation of free radicals that altered the magnitude of the surface charge in the medium emulsions.

The potential gastrointestinal fate of the emulsions was then established by passing them through the INFOGEST *in vitro* digestion model. The properties of the samples were assessed after each phase of the digestion process: mouth, stomach, and small-intestine. In addition, they were analyzed at the initial stages of the small-intestine phase (SI-initial) by adjusting the fluids arising from the end of the stomach phase to pH 7 but without adding the enzymes and bile salts. The SI-initial phase was included because it is the oil droplet size at the beginning of the small-intestine phase that is important for lipid digestion, rather than the original droplet size of the emulsions [32].

Similar to our previous study [18], the mean oil droplet size in all the emulsions remained fairly similar as they moved from the oral phase to the SI-intestinal phase, despite the considerable differences in pH, ionic strength, mucin levels, and enzyme activity in these different gastrointestinal regions (Fig. 2a). The confocal microscopy images were again consistent with the light scattering results (Fig. 3): the droplet size remained fairly constant in each GIT stage. However, the droplet concentration did decrease because the samples were progressively diluted by digestive fluids. Presumably, the good stability of the emulsions to droplet aggregation in the mouth, stomach, and SI-initial phases is because the quillaja saponin remains securely attached to the droplet surfaces and generated strong repulsive interactions.

The absolute value of the ζ-potential decreased significantly ($p < 0.05$) after incubation in the oral phase, ranging from around -40.8 to -21.7 mV, and then further decreased in the stomach phase, ranging from around -9.8 to -2.9 mV (Fig. 2b). These results agree with those of previous studies on related systems [33, 34]. This reduction in the absolute value of the ζ-potential is a result of alterations in the composition and ionization state of the droplet surfaces when the pH, ionic strength, and composition of the GIT fluids are altered. Our results suggest that the adsorbed saponin molecules were able to stabilize the oil droplets against aggregation by generating strong steric repulsion, since extensive droplet aggregation was not observed under conditions where there was only a weak electrostatic repulsion (*i.e.,* a low ζ-potential). When the samples were adjusted to pH 7 at the beginning of the small intestine phase, the absolute value of the negative charge on the particles increased significantly ($p < 0.05$), with the ζ-potential values becoming fairly similar to those in the initial samples, *i.e.,* -63.2 to -55.3 mV (Fig. 3b). This increase in negative charge is due to ionization of the carboxylic acid groups on the quillaja saponin at higher pH values [35]. There were significant differences in the ζ-potentials of different emulsions in the mouth, stomach, and SI-initial phases, but the general trends were similar for all systems.

After intestinal digestion, the physical and structural properties of the emulsions changed greatly. The average particle size of the medium and large emulsions decreased significantly ($p < 0.05$) to 0.31 and 0.71 μm respectively, while that of the fine emulsion remained fairly similar, being around 0.20 μm (Fig. 2a). The confocal microscopy images also showed that the size of the oil-rich particles in the medium and large emulsions decreased after the intestinal phase (Fig. 3). This reduction in particle size is mainly attributed to hydrolysis of the large oil droplets, leading to the generation of digestive products (FFAs and monoacylglycerols). These digestive products then combine with other components within the digestive fluids (*e.g.,* calcium ions, enzymes, bile acids, and mucin) to form a variety of colloidal particles. Some of these particles are readily dispersible in water (*e.g.,* micelles and vesicles), while others are insoluble in water (*e.g.,* calcium soaps, bile salt complexes, protein aggregates, and non-digested oils). In this study, centrifugation was used to separate the soluble fraction (the "mixed micelle" phase) from the insoluble fraction (the "sediment" phase) and undigested fraction (the "oil" phase). The visual appearances of the centrifuged samples are shown in Fig. 2c, which clearly show the sediment and mixed micelle phases. Any non-digested oil phase at the top of the samples was difficult to see because it was very thin.

The size of the particles in the mixed micelle phase was analyzed by dynamic light scattering (Fig. 2d). The average diameter of the mixed micelles in the medium emulsions (218 nm) and large emulsions (191 nm) were significantly ($p < 0.05$) greater than those in the fine emulsions (146 nm). The smaller size of the particles in the fine emulsions may be due to more thorough and complete digestion of the lipid phase. The visible appearance of the mixed micelle phases collected after centrifugation are shown in Fig. 2e. The mixed micelles from the medium emulsions were slightly more turbid than those collected from the fine and large emulsions, which may have been because they contained larger particles that scattered light more strongly (Fig. 2d). Interestingly, the mixed micelles collected from the large emulsions were the least turbid, indicating fewer mixed micelles were present as a consequence of incomplete lipid digestion (Section 3.2).

The average diameters of the colloidal particles in the entire digested intestinal samples (199 to 705 nm) (Fig. 2a), were considerably larger than those in the mixed micelle phase (146 to 218 nm) (Fig. 2d). This effect is due to the removal of the larger insoluble colloidal particles during the centrifugation step used to collect the mixed micelle phase, such as non-digested oil droplets and calcium soaps. It should be noted that different instruments were used for the particle size measurement of these two samples (static and dynamic light scattering), which might also partially account for the observed differences. The static light scattering results showed that the average size of the digested intestinal samples increased with increasing initial oil droplet size, which can again be attributed to the fact that not all of the oil droplets were fully digested for the larger droplets (Fig. 2a). This hypothesis was supported by the confocal microscopy images (Fig. 3), which showed that there were still some non-digested oil droplets in the large emulsions, as well as some other large structures, which were probably insoluble calcium salts and large vesicles.

Previous research has shown that the bioaccessibility of the encapsulated hydrophobic bioactives decreases as the degree of lipid digestion decreases [17]. This effect has been attributed to the fact that some of the hydrophobic bioactives are trapped inside the non-digested oil phase, as well as there are less mixed micelles generated to solubilize them. As a result, a significant fraction of the hydrophobic bioactives is either trapped within the sediment phase or the non-digested lipid phase. The total amount of sediment phase collected after centrifugation of the digested intestinal samples was similar ($p > 0.05$) in all samples, being 1.9, 1.8, 1.7 % for the fine, medium, and large emulsions, respectively (Fig. 2f). This suggests that the initial droplet size did not have a major impact on the amount of sediment formed. Nevertheless, some of the non-digested lipid may have formed a thin layer on the top of the samples.

The ζ-potentials of the colloidal particles in the total digested intestinal samples and in the mixed micelle samples were measured (Fig. 2b). The magnitude of the negative charge significantly ($p < 0.05$) decreased from the beginning (SI-initial) to the end (SI-end) of the intestinal samples (Fig. 2b). This effect is probably because the anionic FFAs generated during lipid digestion dominated the surface charge of the colloidal particles after digestion. Presumably, the surface charge density resulting from these FFAs was considerably less negative than that of the quillaja saponin. In addition, quillaja saponin may be hydrolyzed by the digestive enzymes, thereby reducing its contribution to the surface charge of the lipid droplets [36]. The ζ-potential of the mixed micelles was similar to that measured in the total digested samples, which can again be attributed to the fact that the FFAs dominated the electrical charge measurements.

Overall, these experiments showed that there were considerable differences in the structures and physicochemical properties of the emulsions within different regions of the GIT depending on their initial droplet size.

3.2 Lipid digestion process

Lipid digestion was monitored by titration of the released FFAs. An appreciable fraction of the FFAs from soy oil are long chain ones that are not fully ionized at pH 7 [37], so most of them are not detected by the titration method. As a result, the measured FFA values are much lower at pH 7 than expected (Fig. 4a). For this reason, the samples were titrated to pH 9 using alkaline solution to determine the concentration of FFAs that were not ionized at pH 7. This led to a significant increase in the fraction of FFAs that were detected (Fig. 4a), which agrees with previous studies using the INFOGEST method [25]. Notably, the total amount of FFAs released was greater than 100% after the back titration step was performed, which also agrees with previous research [18]. This effect has been attributed to the fact that some of the monoglycerides were converted to FFAs under strongly alkaline conditions.

The fraction of FFAs released was fairly similar for the fine and medium emulsions at pH 9, being around 124 and 125%, respectively (Fig. 4a). Conversely, the fraction released from the larger emulsions was significantly ($p < 0.05$) lower, being around 99% (Fig. 4a). The kinetics of lipid digestion also depended on droplet size, with the initial digestion rate increasing as the droplet size decreased (Fig. 4b). The specific surface areas of the emulsions increased as their droplet size decreased, so there was a larger number of triacylglycerol molecules exposed to the lipase. Despite the almost ten-fold difference in initial droplet size, the small and medium emulsions appeared to both be fully digested by the end of the small-intestine phase. In contrast, the large emulsions were only partially digested because of their relatively low specific surface area. Nevertheless, human feeding experiments have shown that complete lipid digestion can occur for emulsions containing relatively large oil droplets [38], which can be attributed to the dynamic nature of the human gut. In particular, the transit times of foods within different regions of the GIT can be modulated, as well as the levels of bile and digestive enzymes secreted. As a result, the rate and extent of lipid digestion observed using a simulated GIT may be quite different from that in a real GIT. Clearly, further research is needed to establish *in vitro – in vivo correlations* for different kinds of food matrices.

3.3 Gastrointestinal fate of vitamins A, D, E

In the following section, the impact of oil droplet size and vitamin type on the gastrointestinal fate of the oil soluble vitamins was determined using the *in vitro* digestion model, including their stability, release, transformation, sedimentation, and bioaccessibility. We hypothesized that the molecular characteristics of the vitamins, such as their size and hydrophobicity, would impact their gastrointestinal fate. This hypothesis was based on previous studies that vitamins or pro-vitamins with more hydrophobic structures tend to remain inside non-digested oil droplets, thereby reducing their bioaccessibility [39]. Vitamin A palmitate and vitamin E acetate were used in this study because the esterified forms of these vitamins are typically employed in commercial products in order to improve oxidation stability during storage. All vitamins were added at a level that was 10-fold higher than their recommended daily allowances (RDAs). These relatively high levels were used so that the concentration of the vitamins could be reliably measured using the analytical procedures employed.

3.3.1 Vitamin stability

The resistance of different vitamins to chemical degradation after they had passed through the entire GIT model was measured. It should be noted that conversion of the vitamins from the esterified to non-esterified form was not considered to be degradation in these experiments. Instead, this was considered to be transformation (see later). In all cases, there was an appreciable reduction in the vitamin concentration by the end of the digestion process (Fig. 5). For instance, the percentages of the different vitamins remaining after digestion were around 40-41%, 79-83% and 75-91% for vitamins A, D, and E, respectively, which indicated that vitamin A was the least stable. All the oil-soluble vitamins used in this study are known to be chemically reactive molecules, which are therefore susceptible to chemical degradation. For instance, the retinyl structure of vitamin A contains an electron-dense region that can scavenge free radicals. The hydroxyl groups on the chromane ring of vitamin E have been linked to its strong free radical scavenging activity. The numerous double bonds in vitamin D make it susceptible to oxidation. The elevated temperatures (37 °C), prolonged times (> 4 hours), and high oxygen levels in the GIT model may therefore have promoted the chemical degradation of the oil-soluble vitamins. The samples were covered throughout the GIT experiments to avoid exposure to light, but there was some slight exposure when the samples were transferred from one GIT stage to another, which could also have promoted the chemical degradation of the vitamins. These results suggest that it is important to develop strategies to inhibit the chemical degradation of vitamins within the GIT so as to increase their effectiveness.

The impact of oil droplet size on vitamin stability was also investigated. Droplet size did not significantly impact the stability of vitamins A and D ($p > 0.05$). However, the stability of vitamin E was significantly higher ($p < 0.05$) in the large emulsions (90.7%) than in the fine (74.8%) or medium (78.9%) ones. This may have been because some of the vitamin E was trapped within the non-digested oil phase (Fig. 4), which protected it from oxidation by pro-oxidants in the aqueous phase or interfacial region.

3.3.2 Vitamin bioaccessibility

The bioaccessibility is an important factor contributing to the overall bioavailability of encapsulated vitamins. It depends on two main physicochemical processes: (i) release of the vitamins from the oil droplets; and (ii) solubilization of the vitamins in the mixed micelles ("micellization") [9]. Those vitamins that are not solubilized within the mixed micelles typically end up either in the undigested oil layer (top) or in the sediment layer (bottom) of the digested samples. In this section, we therefore discuss the impact of oil droplet size on the release, sedimentation, and bioaccessibility of each vitamin.

Vitamin A: The concentrations of vitamin A in each phase of the intestinal samples were measured and then the release, sedimentation, and bioaccessibility values were calculated from these data (Fig. 6). As mentioned earlier, the encapsulated vitamins are released from the oil phase upon lipid digestion, and so reducing lipid digestion should reduce the amount of vitamins released. Consequently, the fraction of vitamin A released decreased as the droplet size increased, being around 95.9%, 79.8%, and 51.3% for the fine, medium, and large emulsions respectively (Fig. 6a). Interestingly, the release of the vitamin A from the fine and medium emulsions was substantially different, though the final amount of lipid digested was fairly similar (Fig. 5). This suggests that the kinetics of the digestion process might be critical for the release of the vitamins. In particular, rapid digestion may have led to more efficient transfer of the oil-soluble vitamins from the oil droplets to the mixed micelles. Other studies have also reported that strongly hydrophobic bioactive molecules may not be fully released from large oil droplets that are not fully digested [40].

In our study, the vitamin A palmitate was further hydrolyzed into vitamin A and palmitic acid by the esterases within the pancreatic enzymes. The degree of transformation was relatively high, ranging from 90.3% for the large emulsion to 97.1% for the fine emulsion (Fig. 6a). Notably, the emulsions that gave the highest release of the vitamins (fine emulsions) exhibited the greatest degree of vitamin A transformation, which is probably because the hydrolysis reaction mainly occurs within the aqueous phase, rather than inside the oil droplets [41]. The conversion of the vitamin A to the non-esterified form would also account for its greater degradation, since this form is known to be more susceptible to oxidation. The relative concentrations of vitamin A and vitamin A palmitate in the different phases (total digesta, mixed micelles, and sediment) are shown in Fig. 6b. These results show that there was a greater fraction of the non-esterified form of vitamin A in the mixed micelles than in the sediments, which again can be attributed to the greater exposure of the vitamins to the surrounding aqueous environment when they are solubilized within the micelles.

After the release process, the vitamins were either solubilized to the mixed micelles in the aqueous phase or they formed insoluble complexes that became part of the sediment phase. In this study, most of the released vitamin A (vitamin A and vitamin A palmitate) was located in the micelle phase (Fig. 6b). In the real human gut, this fraction of vitamin A would be available for absorption by the epithelial cells, thereby increasing the overall bioavailability. Vitamin A bioaccessibility decreased with increasing oil droplet size, changing from 86.8% for the fine emulsion to 39.0% of the large emulsions. As mentioned earlier, this can at least partly be attributed to a reduction in the amount of vitamin released from the oil droplets. A relatively small fraction (around 12%) of the vitamin A was also located in the sediment phase for all the emulsions. This vitamin A may have been released from the oil droplets and then precipitated when it came into contact with the aqueous phase, or it may have been solubilized within mixed micelles that were then precipitated by calcium [42]. In the human body, the insoluble fraction of vitamins in the sediment phase would not be expected to be absorbed by the epithelial cells, thereby reducing the bioavailability.

The distribution of vitamin A and vitamin A palmitate within the intestinal samples was somewhat different in the emulsions with different droplet sizes (Fig. 6b). For example, the vitamin A palmitate concentration in the total intestinal sample increased with increasing oil droplet size, changing from 0.8 nmol/ml for the fine emulsions to 2.8 nmol/ml for the large emulsions. This effect is probably because there were more non-digested oil droplets in the large emulsion, which protected the vitamin A palmitate from being converted to the vitamin A form. The total vitamin A palmitate concentration in the sediment phase also increased with increasing oil droplet size, changing from 0.04 to 2.0 nmol/ml. Moreover, the fraction of the vitamin A palmitate in the sediment phase also increased with increasing oil droplet size. This effect is probably because the strongly hydrophobic vitamin A palmitate is more prone to be incorporated into the insoluble structures in the sediment phase.

Vitamin D: The gastrointestinal fate of the vitamin D is shown in Fig 7. Almost complete release of the vitamin was observed for the fine emulsions (93.2%) and medium emulsions (97.4%), while a significantly lower ($p < 0.05$) release was observed for the large emulsions (68.2%) (Fig. 7a). This difference can mainly be attributed to the extent of lipid digestion in different emulsions. As discussed earlier, the fine and medium emulsions were fully digested by the end of the small-intestine phase, whereas the large emulsions were only partially digested (Section 3.2). Consequently, some of the hydrophobic vitamin D molecules remained inside the undigested oil phase in the large emulsions. As a result, the vitamin D bioaccessibility in the fine emulsions (75.8%) and medium emulsions (76.0%) was significantly ($p < 0.05$) higher than in the large emulsions (44.2%) (Fig. 7a). A relatively high bioaccessibility has also been reported in emulsion delivery systems where the lipid phase was completely digested [11].

Similar to vitamin A, the fraction of vitamin D in the sediment phase was higher in the medium emulsions (21.4%) and large emulsions (24.0%) than in the fine emulsions (17.4%) (Fig. 7a). These results suggest that oil droplet size only had a modest influence on the solubilization of the vitamin D within the mixed micelles (micellization). The absolute vitamin D concentrations in each phase were also measured (Fig. 7b), which also showed that there was less vitamin solubilized in the mixed micelles for the large emulsions.

Vitamin E: The gastrointestinal fate of vitamin E is shown in Fig 8. As with the other oil-soluble vitamins, the release of vitamin E decreased significantly ($p < 0.05$) with increasing oil droplet size, from 96.7% for the fine emulsion to 43.1% for the large emulsion (Fig. 8a). The magnitude of the effect was similar to that observed for vitamin A palmitate, since the vitamin E acetate was also strongly hydrophobic (Table 1). A hydrolysis of the vitamin E acetate was detected, but the hydrolysis level was much lower than that reported for vitamin A palmitate, ranging from 2.6 to 3.6% (Fig. 8a). There was no significant ($p > 0.05$) difference in the hydrolysis level among the emulsions containing different oil droplet sizes. The bioaccessibility of vitamin E significantly ($p < 0.05$) decreased with increasing oil droplet size, from 77.4% for the fine emulsion to 20.7% for the large emulsion (Fig. 8a). This effect can partly be due to the reduction in the release of the vitamins from the oil droplets with increasing droplet size discussed above, as well as an appreciable fraction being incorporated into the sediment phase (19-30%). Interestingly, the sediment phase contained much more vitamin E acetate than vitamin E (Fig. 8b). This may be because the non-esterified form of the vitamin E can easily be incorporated into the mixed micelles, whereas the esterified form cannot [43]. In addition, the vitamin E acetate may be more prone to precipitation in the aqueous phase than the vitamin E because it is larger and more hydrophobic.

3.3.3 Impact of vitamin properties on gastrointestinal fate

In this section, we examined the impact of the molecular characteristics of the different vitamins on their gastrointestinal fate in emulsions containing different droplet sizes. The release of the vitamins from the oil droplets appeared to be inversely correlated to their hydrophobicity. Vitamin A palmitate (logP = 14.8) and vitamin E acetate (logP = 10.9) are much more hydrophobic than vitamin D (logP = 7.5) (Table 1). As a result, in the large emulsions that were not fully digested, a much higher fraction of the vitamin A palmitate and vitamin E acetate were not released than for the vitamin D. This result suggests that the most strongly hydrophobic oil-soluble vitamins remained within the hydrophobic core of the oil droplets as the latter were hydrolyzed by lipase. In contrast, for the fine emulsions, which were fully digested by the end of the small-intestine phase, the bioaccessibility was relatively high for all the vitamins because there was no more non-digested oil for the vitamins to remain trapped within.

As discussed earlier, the vitamin A and vitamin E used in this study were initially in an esterified form but were partially hydrolyzed by the digestive enzymes used in the GIT model. The extent of transformation was highly dependent on vitamin type being much higher for vitamin A palmitate than for vitamin E acetate. This difference may be due to a number of physicochemical processes. First, there are differences in the ability of digestive enzymes to hydrolyze different esterified vitamins. For instance, it has been shown that only cholesteryl ester hydrolase can hydrolyze vitamin E acetate [43], whereas several esterases can hydrolyze vitamin A palmitate, including cholesteryl ester hydrolase and pancreatic lipase. Second, the total concentration of vitamin E acetate added was considerably higher (around 10-fold) than vitamin

A palmitate, which might also account its lower transformation. In detail, the total vitamin E concentration in the intestinal phase ranged from 0.24 to 0.29 μmol/ml for all samples (Fig. 8b), whereas the total vitamin A concentration ranged from 0.028 to 0.029 μmol/ml (Fig. 6b). The extent of vitamin transformation in the GIT would be expected to have a major impact on its bioavailability, as it alters the vitamin's molecular weight and hydrophobicity, thereby impacting its tendency to be solubilized in mixed micelles or precipitate into the sediment phase.

The incorporation of the vitamins into the insoluble sediment phase was also influenced by vitamin type. There was considerably less vitamin A (around 12%) in the sediment phase than for vitamin D (around 17-24%) and vitamin E (around 19-30%). This effect may be because the non-esterified form of vitamin A was smaller and less hydrophobic than the other vitamins (Table 1) and was therefore more easily solubilized within mixed micelles rather than the sediment.

Vitamin bioaccessibility depends on the release of the vitamins from the interior of the oil droplets, followed by their solubilization within the mixed micelles. The molecular structure of the oil-soluble vitamins used was shown to influence their bioaccessibility, with the extent of this effect depending on oil droplet size. For the fine emulsions, lipid digestion was complete and the vitamins were fully released. In this case, the bioaccessibility was mainly determined by the ability of the vitamins to be incorporated into the mixed micelles. As a result, the bioaccessibility of vitamin A was much higher than that of vitamin D and vitamin E acetate because the smaller vitamin A molecules could be solubilized more easily. In contrast, for the large emulsions, lipid digestion was incomplete and some of the more hydrophobic vitamins remained trapped within the non-digested oil phase. In particular, vitamin E acetate and vitamin A palmitate showed less release and higher accumulation within the oil droplets. However, there was more vitamin D and vitamin E acetate in the sediment phase than for vitamin A. Overall, vitamin E acetate had the lowest bioaccessibility, followed by vitamin A, then vitamin D for the large emulsions. The vitamin E acetate was also lowest in the medium emulsion, except that the bioaccessibility of vitamin D was higher than that of vitamin A in this case. Lipid digestion of different sized oil droplets might modify the composition or structure of the mixed micelle phase, which then influences the micellization process of the released vitamins. Among all the vitamins, the bioaccessibility of vitamin E acetate was most strongly impacted by the size of the oil droplets in the emulsions. It should be noted that gastric lipase was not included in the *in vitro* digestion simulation used in this study, and hence it would be interesting to include this enzyme in future studies. Moreover, it will be important to assess whether the results obtained from static *in vitro* digestion studies can be correlated to those obtained from *in vivo* feeding studies with animals or humans.

4. Conclusions

The saponin molecules effectively maintained the oil droplet size under the GIT conditions, and the surface charge of the oil droplets depended on the pH of the different GIT regions due to changes in surface composition and ionization. The change of initial oil droplet size did not significantly influence the physical characteristics of the emulsions in the GIT. As expected, the rate of lipid digestion increased with decreasing droplet size because of the increase in specific surface area. After conventional 2 h intestinal phase, complete lipid digestion was measured in the fine and medium emulsions by the end of the small-intestine phase, but not for the large emulsions. Consequently, the release of the hydrophobic vitamins was suppressed in the large emulsions because they tended to accumulate in the non-digested oil phase. These vitamins might be able to be released when long digestion time or higher lipase concentration are used to achieve complete lipid digestion. Moreover, the incorporation of the vitamins into the mixed micelle phase was reduced in the large emulsions, which was attributed to fewer mixed micelles being formed. As a result, more of the vitamins released from the large droplets ended up in the sediment phase after lipid digestion. In combination, these two effects caused the bioaccessibility of the vitamins to decrease with increasing oil droplet size.

The chemical stability of the vitamins in the GIT depended on vitamin type. The vitamin A palmitate was the least stable, followed by vitamin D and vitamin E acetate. Furthermore, vitamin type also impacted the bioaccessibility of the vitamins. For the large emulsions, vitamin D was released more easily from the oil droplets than vitamin E acetate and vitamin A palmitate, which was attributed to it lower hydrophobicity and molar mass. After release, the esterified vitamins were exposed to pancreatic enzymes in the gastrointestinal fluids, which partially hydrolyzed them. Vitamin A palmitate was almost completely hydrolyzed to vitamin A, while most of the vitamin E acetate remained in the esterified form. Vitamin type also impacted the incorporation of the vitamins into the mixed micelles or sediment. More sedimentation and less micelle solubilization were observed for vitamin E acetate and vitamin D than for vitamin A.

Overall, this study highlights the importance of incorporating this type of vitamins within small oil droplets so as to increase their bioaccessibility. It also shows that the molecular characteristics of oil-soluble vitamins influence their bioaccessibility, which should be considered when designing delivery systems. The knowledge generated in this study may be used to design functional foods and supplements with enhanced vitamin bioavailability and therefore efficacy. In the future, it would be recommended to test the impact of vitamin type and droplet size on bioavailability using *in vivo* animal and human feeding studies.

Conflicts of interest

There are no conflicts to declare.

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References

1. D. R. Thomas, Vitamins in aging, health, and longevity, *Clinical interventions in aging*, 2006, **1**, 81.
2. E. M. Wiseman, S. Bar-El Dadon and R. Reifen, The vicious cycle of vitamin a deficiency: A review, *Crit Rev Food Sci Nutr*, 2017, **57**, 3703-3714.
3. T. Beal, E. Massiot, J. E. Arsenault, M. R. Smith and R. J. Hijmans, Global trends in dietary micronutrient supplies and estimated prevalence of inadequate intakes, *PLoS One*, 2017, **12**, e0175554.
4. S. Rautiainen, J. E. Manson, A. H. Lichtenstein and H. D. Sesso, Dietary supplements and disease prevention - a global overview, *Nature reviews. Endocrinology*, 2016, **12**, 407-420.
5. R. Moench-Pfanner, A. Laillou and J. Berger, Introduction: Large-Scale Fortification, an Important Nutrition-Specific Intervention, *Food and nutrition bulletin*, 2012, **33**, S255-S259.
6. D. J. McClements, Development of Next-Generation Nutritionally Fortified Plant-Based Milk Substitutes: Structural Design Principles, *Foods*, 2020, **9**.
7. D. J. McClements, Enhanced delivery of lipophilic bioactives using emulsions: a review of major factors affecting vitamin, nutraceutical, and lipid bioaccessibility, *Food & Function*, 2018, **9**, 22-41.
8. D. J. McClements, F. Li and H. Xiao, The Nutraceutical Bioavailability Classification Scheme: Classifying Nutraceuticals According to Factors Limiting their Oral Bioavailability, *Annu Rev Food Sci Technol*, 2015, **6**, 299-327.
9. C. Dima, E. Assadpour, S. Dima and S. M. Jafari, Bioavailability of nutraceuticals: Role of the food matrix, processing conditions, the gastrointestinal tract, and nanodelivery systems, *Comprehensive Reviews in Food Science and Food Safety*, 2020, **19**, 954-994.
10. P. Borel, D. Preveraud and C. Desmarchelier, Bioavailability of vitamin E in humans: an update, *Nutr Rev*, 2013, **71**, 319-331.
11. Y. Tan, J. Liu, H. Zhou, J. M. Mundo and D. J. McClements, Impact of an indigestible oil phase (mineral oil) on the bioaccessibility of vitamin D3 encapsulated in whey protein-stabilized nanoemulsions, *Food Res. Int.*, 2019, **120**, 264-274.
12. L. Salvia-Trujillo, S. H. Verkempinck, L. Sun, A. M. Van Loey, T. Grauwet and M. E. Hendrickx, Lipid digestion, micelle formation and carotenoid bioaccessibility kinetics: Influence of emulsion droplet size, *Food Chemistry*, 2017, **229**, 653-662.
13. J. M. Aguilera, The food matrix: implications in processing, nutrition and health, *Crit Rev Food Sci Nutr*, 2019, **59**, 3612-3629.
14. A. Helbig, E. Silletti, E. Timmerman, R. J. Hamer and H. Gruppen, In vitro study of intestinal lipolysis using pH-stat and gas chromatography, *Food Hydrocolloids*, 2012, **28**, 10-19.
15. M. Golding and T. J. Wooster, The influence of emulsion structure and stability on lipid digestion, *Current Opinion in Colloid & Interface Science*, 2010, **15**, 90-101.
16. D. J. McClements, *Food emulsions: principles, practices, and techniques*, CRC press, 2015.

17. L. Salvia-Trujillo, C. Qian, O. Martin-Belloso and D. J. McClements, Influence of particle size on lipid digestion and beta-carotene bioaccessibility in emulsions and nanoemulsions, *Food Chemistry*, 2013, **141**, 1472-1480.
18. Y. Tan, Z. Zhang, J. Liu, H. Xiao and D. J. McClements, Factors impacting lipid digestion and nutraceutical bioaccessibility assessed by standardized gastrointestinal model (INFOGEST): oil droplet size, *Food & Function*, 2020, **11**, 9936-9946.
19. K. Ahmed, Y. Li, D. J. McClements and H. Xiao, Nanoemulsion- and emulsion-based delivery systems for curcumin: Encapsulation and release properties, *Food Chemistry*, 2012, **132**, 799-807.
20. M. Yao, Z. Li, D. Julian McClements, Z. Tang and H. Xiao, Design of nanoemulsion-based delivery systems to enhance intestinal lymphatic transport of lipophilic food bioactives: Influence of oil type, *Food Chem*, 2020, **317**, 126229.
21. L. Mutsokoti, A. Panozzo, A. Pallares Pallares, S. Jaiswal, A. Van Loey, T. Grauwet and M. Hendrickx, Carotenoid bioaccessibility and the relation to lipid digestion: A kinetic study, *Food Chem*, 2017, **232**, 124-134.
22. M. E. Carlotti, V. Rossatto and M. Gallarate, Vitamin A and vitamin A palmitate stability over time and under UVA and UVB radiation, *International journal of pharmaceutics*, 2002, **240**, 85-94.
23. A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assuncao, S. Ballance, T. Bohn, C. Bourlieu-Lacanal, R. Boutrou, F. Carriere, A. Clemente, M. Corredig, D. Dupont, C. Dufour, C. Edwards, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. R. Mackie, C. Martins, S. Marze, D. J. McClements, O. Menard, M. Minekus, R. Portmann, C. N. Santos, I. Souchon, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies and I. Recio, INFOGEST static in vitro simulation of gastrointestinal food digestion, *Nature Protocols*, 2019, **14**, 991-1014.
24. Y. Tan, Z. Zhang, H. Zhou, H. Xiao and D. J. McClements, Factors impacting lipid digestion and β-carotene bioaccessibility assessed by standardized gastrointestinal model (INFOGEST): oil droplet concentration, *Food & Function*, 2020, **11**, 7126-7137.
25. Y. Tan, R. Li, H. Zhou, J. Liu, J. M. Mundo, R. Zhang and D. J. McClements, Impact of calcium levels on lipid digestion and nutraceutical bioaccessibility in nanoemulsion delivery systems studied using standardized INFOGEST digestion protocol, *Food & Function*, 2020, **11**, 174-186.
26. Y. Li and D. J. McClements, New mathematical model for interpreting pH-stat digestion profiles: impact of lipid droplet characteristics on in vitro digestibility, *Journal of Agricultural and Food Chemistry*, 2010, **58**, 8085-8092.
27. K. Yoshida, T. Sekine, F. Matsuzaki, T. Yanaki and M. Yamaguchi, Stability of vitamin A in oil-in-water-in-oil-type multiple emulsions, *Journal of the American Oil Chemists' Society*, 1999, **76**, 1-6.
28. A. Abbasi, Z. Emam-Djomeh, M. A. Mousavi and D. Davoodi, Stability of vitamin D(3) encapsulated in nanoparticles of whey protein isolate, *Food Chemistry*, 2014, **143**, 379-383.
29. S. Lv, J. Gu, R. Zhang, Y. Zhang, H. Tan and D. J. McClements, Vitamin E encapsulation in plant-based nanoemulsions fabricated using dual-channel

microfluidization: Formation, stability, and bioaccessibility, *Journal of Agricultural and Food Chemistry*, 2018, **66**, 10532-10542.

30. B. Öztürk, Nanoemulsions for food fortification with lipophilic vitamins: Production challenges, stability, and bioavailability, *European Journal of Lipid Science and Technology*, 2017, **119**.

31. S. Ariyaprakai and S. R. Dungan, Influence of surfactant structure on the contribution of micelles to Ostwald ripening in oil-in-water emulsions, *J Colloid Interface Sci*, 2010, **343**, 102-108.

32. Y. Tan and D. J. McClements, Improving the bioavailability of oil-soluble vitamins by optimizing food matrix effects: A review, *Food Chemistry*, 2021, **348**.

33. Y. Yang, H. Xiao and D. J. McClements, Impact of Lipid Phase on the Bioavailability of Vitamin E in Emulsion-Based Delivery Systems: Relative Importance of Bioaccessibility, Absorption, and Transformation, *J Agric Food Chem*, 2017, **65**, 3946-3955.

34. Y. Tan, Z. Zhang, J. Muriel Mundo and D. J. McClements, Factors impacting lipid digestion and nutraceutical bioaccessibility assessed by standardized gastrointestinal model (INFOGEST): Emulsifier type, *Food Res. Int.*, 2020, **137**.

35. Y. Yang, M. E. Leser, A. A. Sher and D. J. McClements, Formation and stability of emulsions using a natural small molecule surfactant: Quillaja saponin (Q-Naturale®), *Food Hydrocolloids*, 2013, **30**, 589-596.

36. J. Navarro del Hierro, T. Herrera, T. Fornari, G. Reglero and D. Martin, The gastrointestinal behavior of saponins and its significance for their bioavailability and bioactivities, *Journal of Functional Foods*, 2018, **40**, 484-497.

37. P. Sassene, K. Kleberg, H. D. Williams, J. C. Bakala-N'Goma, F. Carriere, M. Calderone, V. Jannin, A. Igonin, A. Partheil, D. Marchaud, E. Jule, J. Vertommen, M. Maio, R. Blundell, H. Benameur, C. J. H. Porter, C. W. Pouton and A. Mullertz, Toward the Establishment of Standardized In Vitro Tests for Lipid-Based Formulations, Part 6: Effects of Varying Pancreatin and Calcium Levels, *The AAPS Journal*, 2014, **16**, 1344-1357.

38. M. Armand, B. Pasquier, M. Andre, P. Borel, M. Senft, J. Peyrot, J. Salducci, H. Portugal, V. Jaussan and D. Lairon, Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract, *Am. J. Clin. Nutr.*, 1999, **70**, 1096-1106.

39. C. Sy, B. Gleize, O. Dangles, J. F. Landrier, C. C. Veyrat and P. Borel, Effects of physicochemical properties of carotenoids on their bioaccessibility, intestinal cell uptake, and blood and tissue concentrations, *Mol Nutr Food Res*, 2012, **56**, 1385-1397.

40. H. T. Nguyen, M. Marquis, M. Anton and S. Marze, Studying the real-time interplay between triglyceride digestion and lipophilic micronutrient bioaccessibility using droplet microfluidics. 1 lab on a chip method, *Food Chemistry*, 2019, **275**, 523-529.

41. P. Borel, B. Pasquier, M. Armand, V. Tyssandier, P. Grolier, M.-C. Alexandre-Gouabau, M. Andre, M. Senft, J. Peyrot and V. Jaussan, Processing of vitamin A and E in the human gastrointestinal tract, *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 2001, **280**, G95-G103.

42. J. Corte-Real and T. Bohn, Interaction of divalent minerals with liposoluble nutrients and phytochemicals during digestion and influences on their bioavailability - a review, *Food Chemistry*, 2018, **252**, 285-293.

43. C. Desmarchelier, F. Tourniaire, D. P. Preveraud, C. Samson-Kremser, I. Crenon, V. Rosilio and P. Borel, The distribution and relative hydrolysis of tocopheryl acetate in the different matrices coexisting in the lumen of the small intestine during digestion could explain its low bioavailability, *Mol Nutr Food Res*, 2013, **57**, 1237-1245.

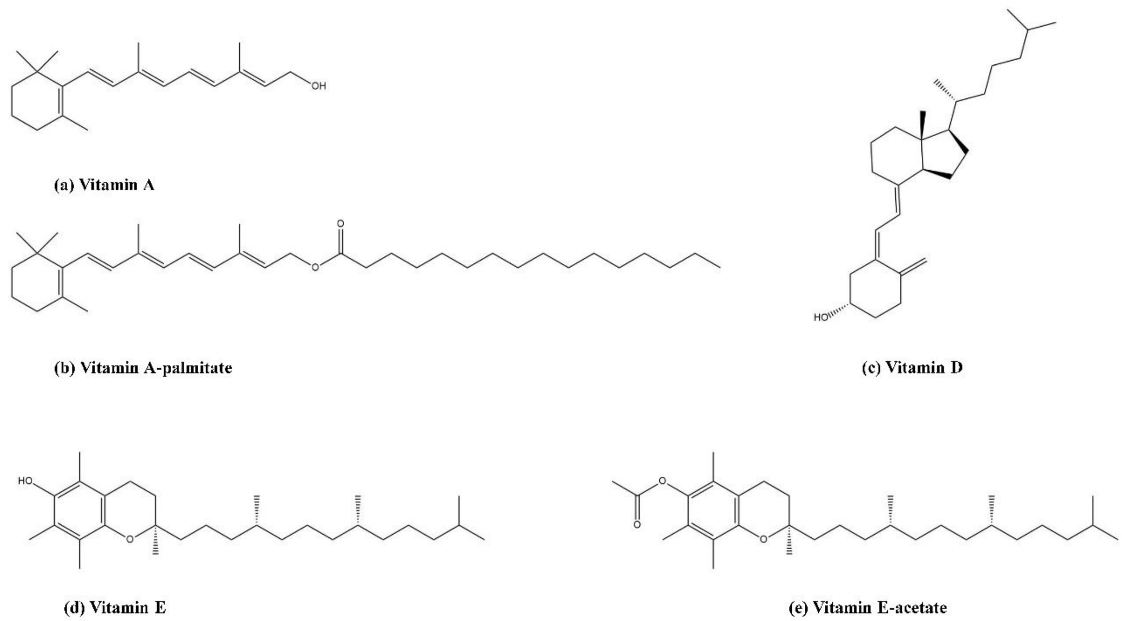

Fig. 1. The chemical structure of oil soluble vitamins. The data were obtained from National Center for Biotechnology Information, U.S. National Library of Medicine.

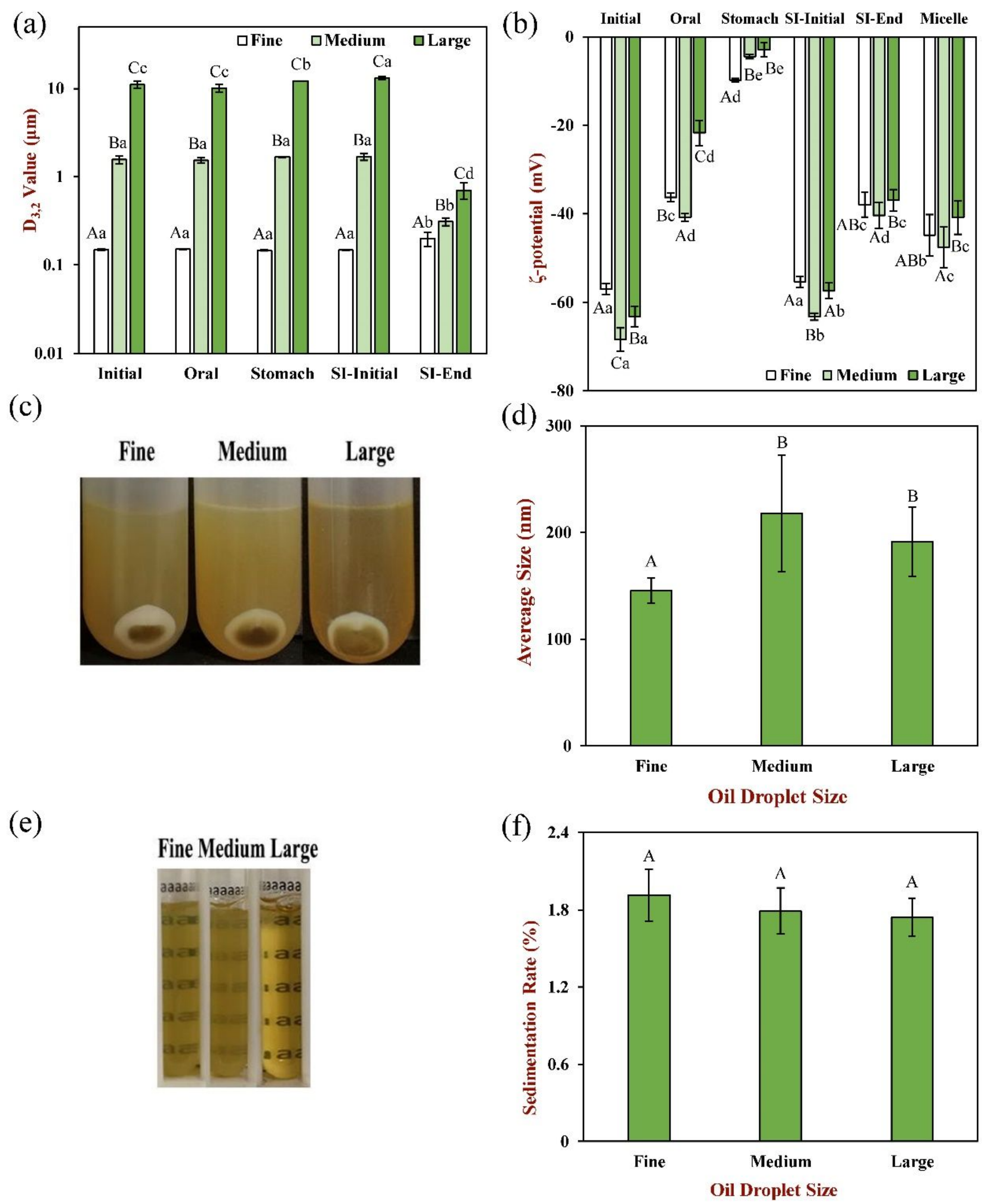

Fig. 2. Physical and structural properties of soy oil-in-water emulsions with different initial oil droplet diameters during passage through a simulated GIT: (a) Mean particle diameter ($D_{3,2}$); (b) ζ-potential; (c) photographs of samples after digestion and centrifugation; (d) mean particle size (Z-average diameter) of mixed micelles measured by dynamic light scattering; (e) photographs of mixed micelles; and (f) sedimentation fraction of intestinal samples. Different capital letters (A, B, C) designate significant differences ($p < 0.05$) among emulsion samples with different oil droplet size (same GIT stage), while lower-case letters (a, b, c) designate different stages (same oil droplet size).

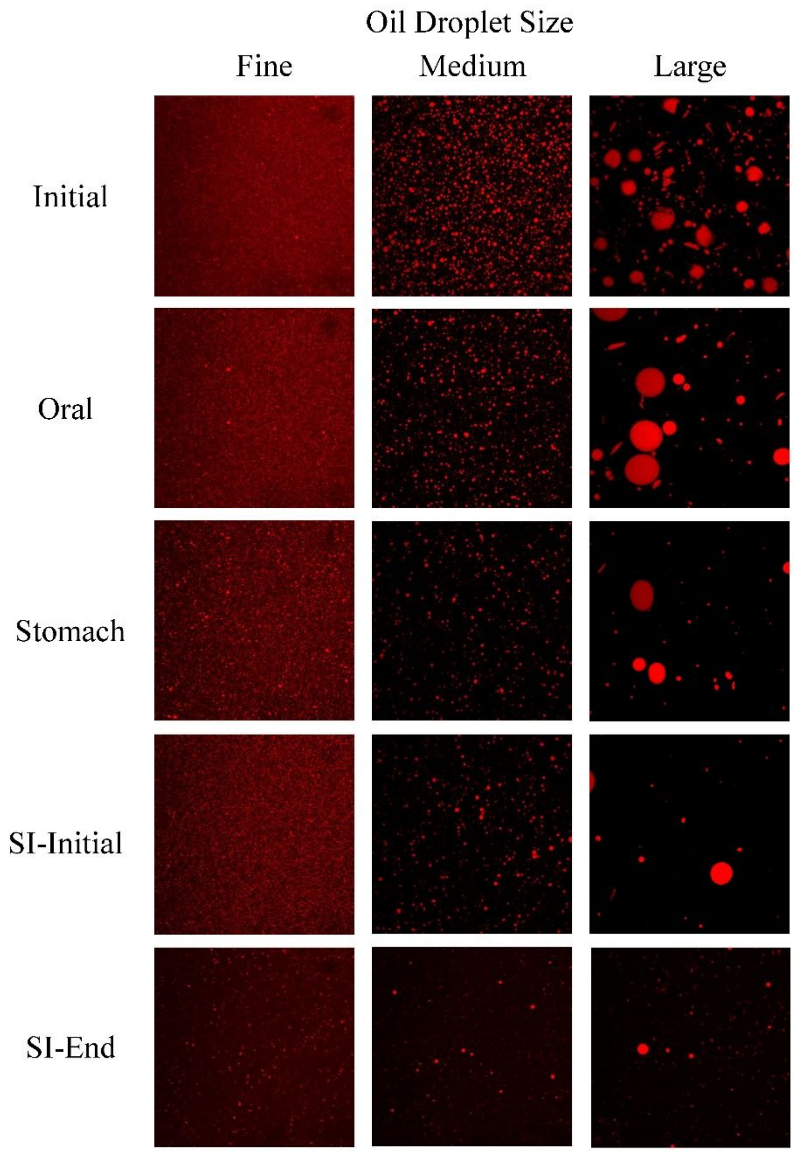

Fig. 3. The confocal microscopy photos of soy oil in water emulsions with different oil droplet size during digestion.

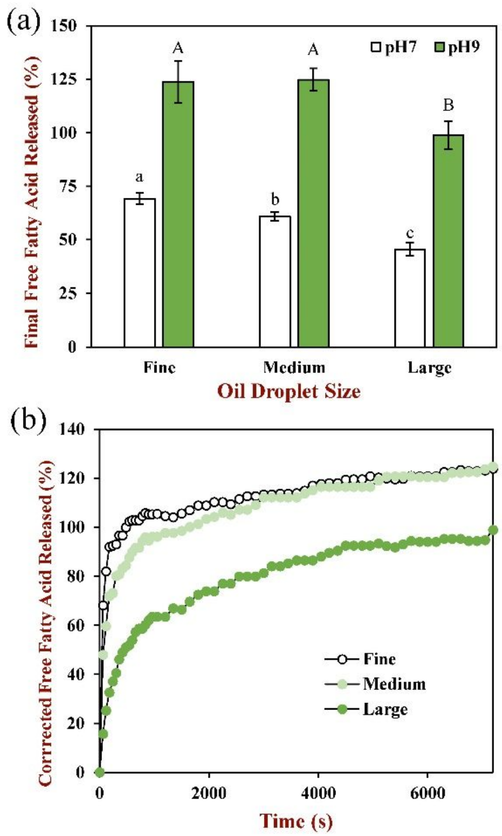

Fig. 4. The impact of oil droplet size on the final degree (a) and rate (b) of free fatty acid release of soy oil in water emulsions during the intestinal phase. Capital letters (A, B, C) and lower-case letters (a, b, c) were used to designate significant difference ($p < 0.05$) among different oil droplet size.

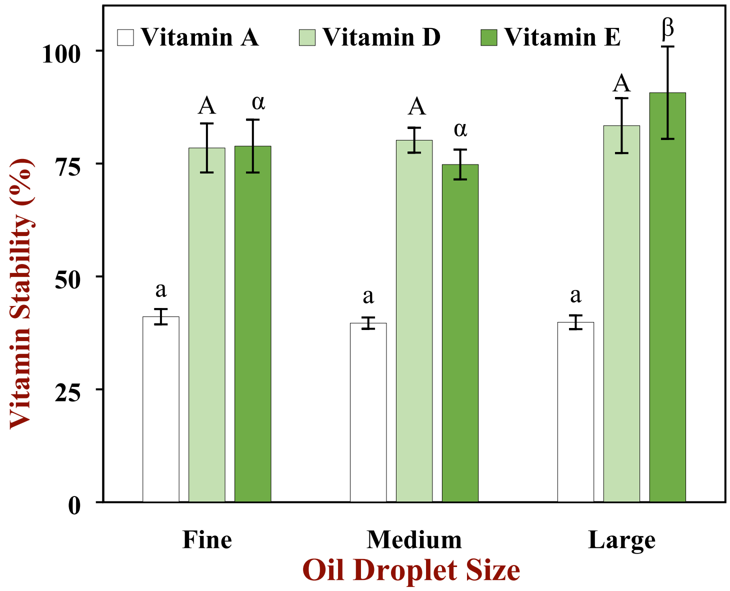

Fig. 5. The stability of vitamins A, D, and E encapsulated in soy oil-in-water emulsions with different initial oil droplet diameters at the end of the in vitro digestion model. Capital letters (A, B, C), lower-case letters (a, b, c) and Greek letters (α, β, γ) were used to designate significant differences ($p < 0.05$) among different oil droplet sizes.

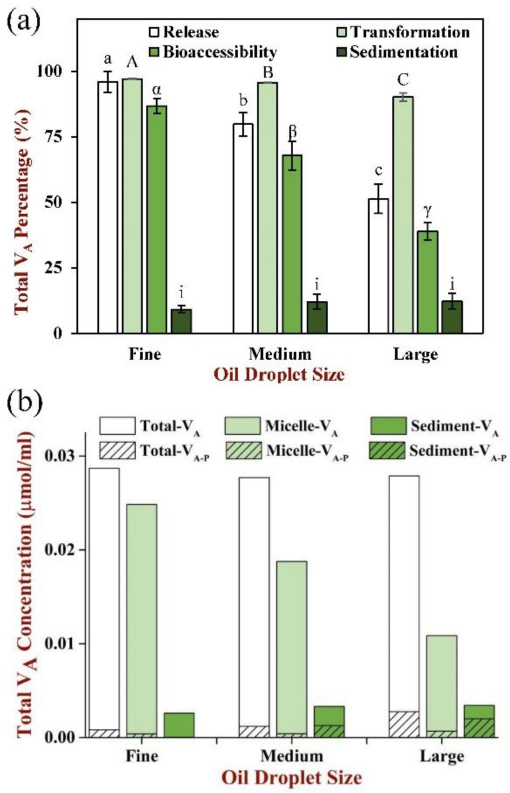

Fig. 6. The effect of oil droplet size on (a) the release, transformation, bioaccessibility, and sedimentation, and (b) the vitamin A and vitamin A-palmitate (total vitamin A) concentration in each phase of the soy oil-in-water emulsions after in vitro digestion. Capital letters (A, B, C), lower-case letters (a, b, c), the Greek letters (α, β, γ) and Roman numbers (i, ii, iii) were used to designate significant difference ($p < 0.05$) among different oil droplet size.

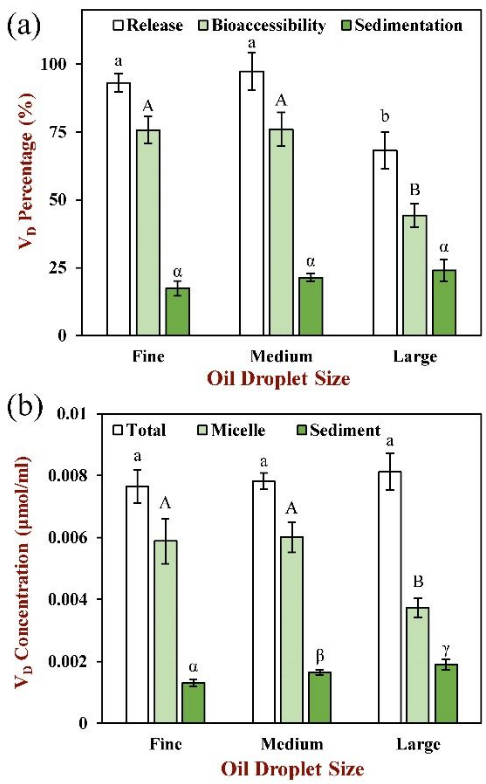

Fig. 7. The effect of oil droplet size on (a) the release, bioaccessibility, and sedimentation, and (b) vitamin D concentration in each phase of the soy oil-in-water emulsions after in vitro digestion. Capital letters (A, B, C), lower-case letters (a, b, c) and the Greek letters (α, β, γ) were used to designate significant difference ($p < 0.05$) among different oil droplet size.

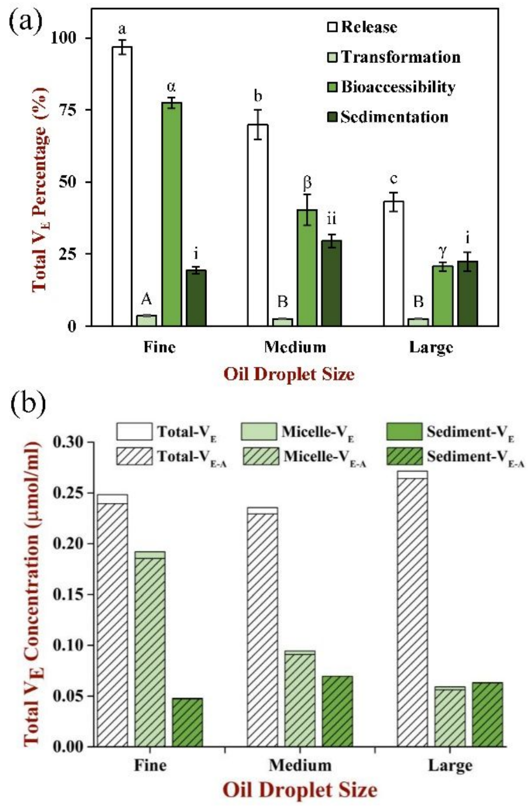

Fig. 8. The effect of oil droplet size on (a) the release, transformation, bioaccessibility, and sedimentation, and (b) vitamin E and vitamin E-acetate (total vitamin E) concentrations in each phase of the soy oil-in-water emulsions after in vitro digestion. Capital letters (A, B, C), lower-case letters (a, b, c), the Greek letters (α, β, γ) and Roman numbers (i, ii, iii) were used to designate significant difference ($p < 0.05$) among different oil droplet size.

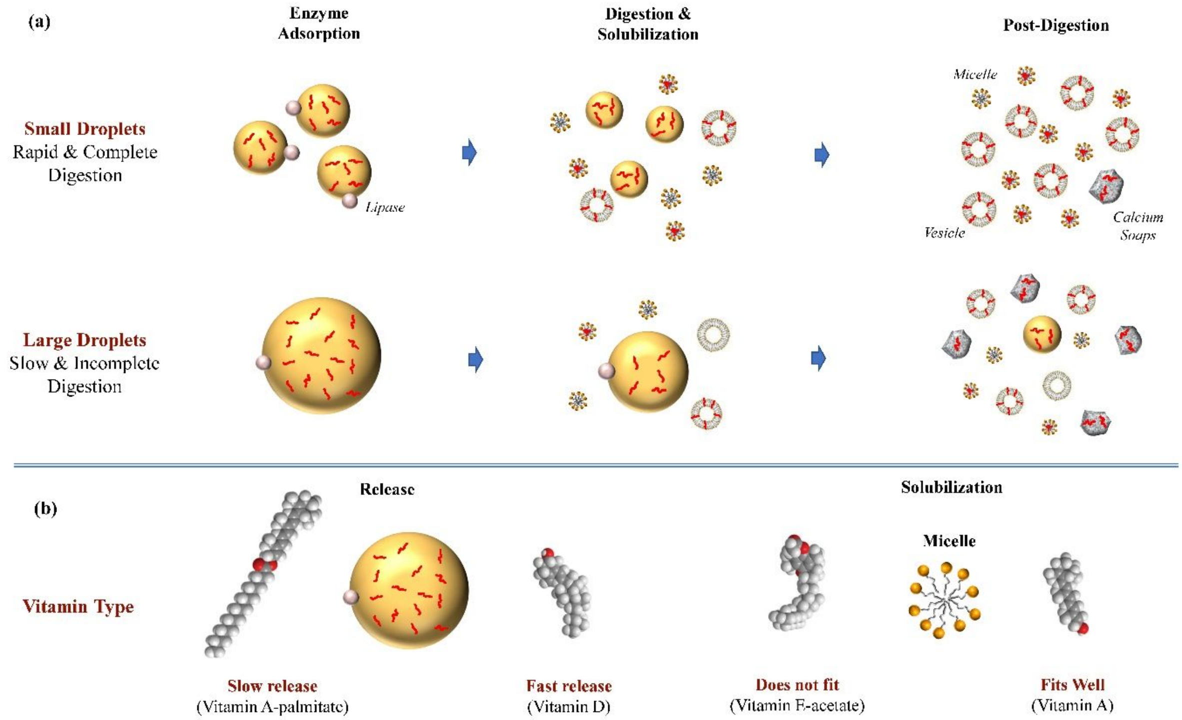

Fig. 9. The Schematic diagram describing the impact of oil droplet size and vitamin type on the bioaccessibility of oil soluble vitamins: (a) a large oil droplet size reduces enzyme adsorption to the interface, inhibits lipid digestion process, and inhibits the release of vitamins; and it also influences the vitamin micellization process; (b) the size and hydrophobicity of the vitamins influence their release from the oil droplets and the solubilization into the micelle structures. The 3D structure of the oil soluble vitamins was obtained from National Center for Biotechnology Information, U.S. National Library of Medicine.