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Fabrication of plant-based vitamin D_3-fortified nanoemulsions: Influence of carrier oil type on vitamin bioaccessibility

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Abstract

The influence of carrier oil type (corn, fish, or flaxseed oil) on the production, stability, and simulated gastrointestinal behavior of vitamin–fortified nanoemulsions was studied. The nanoemulsions were formulated using pea protein as an emulsifier since there is increasing interest in substituting artificial and animal-based food ingredients with natural plant-based alternatives. Lipid digestion and vitamin D_3 bioaccessibility were measured when the nanoemulsions were subjected to a three-stage *in vitro* gastrointestinal tract: oral, gastric, and small intestinal. The majority of all three lipids were digested within the first few minutes in the simulated small intestine, with the corn oil nanoemulsions being digested faster than the fish or flaxseed oils. Moreover, a greater fraction of triglycerides were digested by the end of the small intestine for the corn oil than for the fish and flaxseed oils. For the different carrier oils, vitamin bioaccessibility was ranked: corn oil > flaxseed oil ≈ fish oil. These results suggest that monounsaturated-rich oils (such as corn oil) are better for encapsulating and delivering vitamin D_3 than polyunsaturated-rich ones (such as flaxseed or fish oil). The insights gained here may aid in the formulation of more efficacious vitamin-fortified foods and beverages from plant-derived ingredients.

Keywords: Nanoemulsions; Vitamin D; Gastrointestinal fate; Bioaccessibility; Bioavailability

Introduction

Oil-in-water nanoemulsions are particularly effective oral delivery vehicles for hydrophobic bioactives, such as non-polar antimicrobials, antioxidants, colors, flavors, nutraceuticals, and vitamins.[1-5] These colloidal dispersions contain relatively small (radius < 100 nm) emulsifier-coated lipid droplets dispersed within water, making them particularly suitable for use in fluid products with aqueous continuous phases.[6, 7] Moreover, they can be transformed into powders using commercial dehydration technologies such as spray drying, thereby allowing them to be successfully introduced into dried foods too.[8] Numerous types of synthetic, natural, and nature-derived emulsifiers can be used to coat the oil droplets in food nanoemulsions, but there is a growing emphasis on the identification of plant-based emulsifiers because of their perceived health, environmental, and sustainability benefits.[9] For this reason, researchers have recently characterized and compared the performance of different kinds of plant-based emulsifiers for producing and stabilizing emulsions, including proteins, polysaccharides, phospholipids, and biosurfactants.[10]

Oil-in-water nanoemulsions are ideally suited for the encapsulation and delivery of fat-soluble vitamins, such as vitamins A, D, E, and K, because the small oil droplets are quickly digested within the human gastrointestinal tract (GIT), thereby rapidly generating mixed micelles capable of solubilizing and transporting them.[11] *In vitro* and *in vivo* studies have shown that food-grade nanoemulsions produced by microfluidization can greatly enhance the bioaccessibility and absorption of vitamin D.[12] Nanoemulsions produced using high-intensity sonication also proved to be effective at encapsulating vitamin D and increasing its *in vitro* bioaccessibility.[13] Nanoemulsions generated using high-pressure homogenization were shown to enhance the antioxidant activity of vitamins A and E.[14] Animal feeding studies have shown that incorporation of vitamin E in nanoemulsions increased its bioavailability 3-fold compared to conventional emulsions, which was probably because the rapid hydrolysis of the triglycerides led to rapid release and solubilization of the vitamins.[15]

As well as improving the bioavailability and bioactivity of oil-soluble vitamins, nanoemulsions also offer a convenient vehicle for incorporating them within commercial foods and beverages.[16] As mentioned earlier, nanoemulsions can be fabricated in either fluid or powdered forms that are suitable for incorporation into products that are liquid, semi-solid, or solid. Nanoemulsions can also be created that are transparent, cloudy, or opaque by varying their particle size distribution, which means they can be incorporated into either clear or opaque products. Typically, the mean particle diameter must be less than about 50 nm for the nanoemulsions to become clear, which requires careful optimization of system

composition and homogenization conditions.[17, 18] Finally, nanoemulsions typically have good long-term stability because they are more resistant to creaming and aggregation than conventional emulsions.[19] All of these characteristics make nanoemulsions advantageous for certain commercial applications.

The main objective of the present research was to study the influence of carrier oil type on the behavior of vitamin D_3-fortified nanoemulsions under simulated GIT conditions. Vitamin D_3 is a non-polar micronutrient that is critical for human health and wellbeing, but which is currently under-consumed in many developed and developing countries because it is not naturally present in many foods.[20] Vitamin D_3 can be synthesized in our bodies when the ultraviolet rays in sunlight strike out skin, but this source may be limited in people who live in regions of the world with low sunlight or who are usually protected by clothes or sunscreen. Vitamin D_3 deficiency can result in osteoporosis, weak bones, growth retardation, and muscle weakness.[21] There is, therefore, great interest in the fortification of foods and beverages with this essential micronutrient. However, this can be challenging due to its poor water-solubility, high sensitivity to degradation, and limited bioavailability.[22]

We hypothesized that encapsulation of vitamin D_3 within carefully designed nanoemulsions could overcome these challenges. Moreover, we co-encapsulated the vitamin with carrier oil sources that were rich in either monounsaturated (corn oil) or polyunsaturated (fish or flaxseed oils) fatty acids. Previously, it has been shown that co-ingestion of vitamin D with omega-3 enriched oils led to synergistic health benefits.[23] In this study, we compared the behavior of an animal-based (fish oil) and a plant-based (flaxseed oil) source of omega-3 fatty acids. Moreover, we used a plant-based emulsifier (pea protein) to formulate the nanoemulsions. Ultimately, we aimed to determine whether a fully plant-based nanoemulsion system could be developed to deliver vitamin D_3, as this may increase its utilization in vegan and vegetarian diets.

Materials and Methods

Materials

Fish oil was obtained from DSM Nutritional Products Ltd. (Columbia, MD). Flaxseed oil (International Collection, AAK, Newark, NJ) and corn oil (Mazola, ACH Food Companies, Inc., Memphis, TN) were purchased from a local supermarket. Pea protein (Harvest Pro 85C) was kindly donated by Glanbia Nutritionals (Fitchburg, WI). The supplier reported that the total protein content of this ingredient was 84.2 wt%. Vitamin D_3 was purchased from BASF

(Ludwigshafen am Rhein, Germany). The vitamin was dissolved in medium-chain-triglycerides at a concentration of 1.0 million IU/g. All other chemicals were of analytical grade and obtained from Thermo Fisher Scientific Inc. (Waltham, MA) or Sigma-Aldrich (St. Louis, MO). Double distilled water was utilized in the preparation of all solutions.

Protein Solution Preparation

In a preliminary experiment, we observed that after dispersion in buffer solution (5 mM phosphate, pH 7), the pea proteins formed cloudy dispersions with some evidence of sedimentation. For this reason, various procedures were investigated to remove the insoluble matter from the pea protein solutions prior to use: heating to different temperatures (25, 40, or 45 °C); sonication in an ultrasonic water bath (for 60 min); or centrifugation (14,000 g for 15 or 60 min). After treatment, the protein solutions were filled into petri-dishes and frozen at -80 °C. The frozen protein samples were then freeze-dried overnight (Virtis Company, Gardiner, NY, United States) and then the dried samples were ground to a powder.

The protein content was measured using the standard Lowry assay, using bovine serum albumin as a standard to prepare the calibration curve.

Emulsion Preparation

Oil-in-water emulsions were prepared using 90 % w/w aqueous phase and 10 % w/w oil phase. The oil phase consisted of 99 % w/w flaxseed oil, corn oil, or fish oil, in which 1 % w/w vitamin D_3-enriched oil was added. Aqueous phases were prepared containing various levels of pea protein dispersed in buffer solution (5 mM phosphate, pH 7) and then stored overnight at 4 °C. Afterwards, the pea protein solution was centrifuged for 15 minutes at 14,000 g. The pH values were readjusted, if required.

A two-step procedure was used for homogenization. First, the two phases were blended together using a high-shear blender (Bamix, BSP 70512, Mettlen, Switzerland) for two minutes at room temperature. Second, the resulting coarse emulsion was passed five times through a high-pressure homogenizer (Microfluidics M110L, Newton, MA, USA) at 12,000 psi. A cooling coil immersed in ice water was used to control the temperature of the samples during homogenization.

Particle Characterization

The particle size characteristics were measured by static light scattering (Mastersizer 3000, Malvern, Westborough, MA, USA). The particle charge (ζ-potential) was measured

using laser Doppler micro-electrophoresis (Zetasizer Nano-ZS, Zen3600, MAL500473, Malvern, Westborough, MA, USA). Samples were diluted using buffer solutions with the same pH as the samples being analyzed to avoid multiple scattering. All measurements were conducted at room temperature on the day of preparation. To reduce statistical errors, they were repeated twice for each duplicate sample.

Microstructural analysis

A confocal fluorescent microscope (Nikon, D-Eclipse C1, Tokyo, Japan) with a 10× eye piece lens and a 60× (oil immersion) objective lens was used for taking images of the samples. Nile-red was used for staining the fat droplets.

***In vitro* digestion**

The vitamin-loaded nanoemulsions were passed through a simulated GIT that has been described in detail elsewhere,[24] and so is only described briefly here. All procedures were carried out at 37 °C and all solutions were preheated to this temperature prior to use.

Mouth Stage. 20 mL of diluted nanoemulsions (2% w/w oil content) and 20 mL of artificial saliva (containing mucin and minerals) were added to a 100-mL-beaker, the mixture was adjusted to pH 6.8, and then swirled using a shaker for two minutes (100 rpm).

Stomach Stage. 20 mL of sample from the mouth phase was mixed with 20 mL of artificial gastric fluid (containing pepsin, salts, and acids), the mixture was adjusted to pH 2.5, and then swirled using a shaker for two hours (100 rpm).

Intestine Stage. 30 mL of sample from the stomach phase was transferred into a 100-mL-beaker and placed into a water bath. The sample was then adjusted to pH 7.0 using HCl and NaOH solutions. Afterwards, 1.5 mL salt solution, 3.5 mL bile salt solution, and 2.5 mL lipase solution were sequentially added and the automatic titration unit was started. The release of free fatty acids was then determined by measuring the amount of alkaline solution (0.25 N NaOH) required to maintain a constant neutral pH as described previously.[25]

The particle size, charge, and microstructure of the samples was measured after exposure to each step in the simulated GIT.

The lipid digestion profiles within the small intestine phase were measured by measuring the amount of NaOH solution titrated into the samples to neutralize any free fatty acids generated:[26]

$$FAA\ (\%) = 100 \times (V_{NaOH} \times m_{NaOH} \times \mathrm{M}_{lipid}) / (W_{lipid} \times 2) \qquad (1)$$

V_{NaOH} is the volume of NaOH solution needed to neutralize the FFAs generated (in L), m_{NaOH} is the molarity of the sodium hydroxide solution (in mols L^{-1}), M_{lipid} is the molecular weight of the oil (in g/mol), and W_{lipid} is the weight of the oil in the reaction chamber (in g). The molecular weights of the lipids were 872, 872, and 904 g/mol for corn, flaxseed, and fish oil, respectively, which were calculated from their fatty acid compositions (supplementary information).

It should be noted that the simple simulated GIT method used in our study does not include all of the enzymes and other gastrointestinal constituents found in the real human gut, such as amylase in the mouth or trypsin/chymotrypsin in the small intestine. Nevertheless, it can still serve as a rapid screening tool to provide valuable information about the potential gastrointestinal fate of the different formulations used.

Bioaccessibility of Vitamin D_3

Vitamin D_3 bioaccessibility of each sample was measured after passing through the simulated GIT according to a method established previously.[27] Briefly, 20 mL of the small intestine digest was centrifuged (Sorvall Lynx 4000 Centrifuge, Thermo Scientific, Agawam, MA, United States) at 18,000 rpm and 4 °C for 30 min. This process typically resulted in the separation of the samples into three phases: an opaque sediment; a clear middle phase; an oily upper phase. The clear middle phase was assumed to consist of mixed micelles containing the solubilized vitamin. The bioaccessibility was calculated from the vitamin D_3 concentrations measured in the micelle phase ($C_{Micelle}$) and whole digest (C_{Digest}) using the following equation:

$$Bioaccessibility = 100 \times \frac{C_{Micelle}}{C_{Digest}} \quad (2)$$

Vitamin D_3 Extraction and HPLC Analysis. The vitamin D extraction and HPLC analysis methods were adapted from those described in a previous study[28] with some modifications. 2 mL of the micelle phase or small intestine digest were mixed with 4 mL of hexane-ethanol (1:1, v/v) extraction solution. The mixture was vigorously vortexed and then centrifuged (ThermoFisher Scientific, Sorvall ST8, Waltham, MA, USA) at 2000 rpm for 2 min. Afterwards, the supernatant was collected and dried under a nitrogen atmosphere. 1 mL of methanol was added into the dried sample to dissolve the vitamin D_3 and then the resulting solution was filtered through a 0.45 μm filter (VWR International, Philadelphia, PA, USA) for HPLC analysis. The HPLC system (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA) was equipped with an inline degasser, a binary pump, a variable wavelength

detector (VWD), an auto-sampler, and a column temperature controller. Chromatographic analysis was performed with a RP-HPLC column (Zorbax SB-C18, 250 mm x 4.6 mm id, 5 μm) as the stationary phase and methanol:water (95:5) as the mobile phase. The flow rate was set at 1 mL/min and the column temperature was 25 °C. The injection volume was 20 μL. The detection wavelength was 265 nm. Data storage and analysis was carried out using the instrument software (Agilent ChemStation).

Data Analysis

At least two freshly prepared samples were applied for each measurement. The data were expressed as means and standard deviations from two measurements made on at least two replicates. Statistical analysis was performed using a statistical software package (SPSS) and the means were subject to Duncan's test. *P*-value <0.05 was considered to be statistically significant throughout the study.

Results and Discussion

Several previous studies have already shown that carrier oil type can influence the bioaccessibility of hydrophobic nutraceuticals encapsulated within nanoemulsion-based delivery systems.[27, 29] The main aim of current study was to gain a better insight of how carrier oil type impacted the bioaccessibility of vitamin D_3 encapsulated in plant-based nanoemulsions formulated using pea protein as an emulsifier.

Preparation of protein solutions

Protein-based emulsifiers usually have to solubilized in the aqueous phase before they can manifest their emulsifying abilities. The commercial pea protein used in this study had a relatively poor solubility profile when dispersed directly in buffer solution (pH 7) at ambient temperature. For this reason, alternative preparation procedures were examined to enhance its solubility characteristics. When pea proteins were incubated overnight at 4 °C in buffer solution they produced opaque, inhomogeneous dispersions, in which a part of the protein precipitated. The emulsions prepared from these solutions were relatively unstable and tended to cream rapidly. Different treatments were therefore examined for their potential to enhance the water-solubility and emulsification properties of the pea proteins. The influence of 15 min centrifugation, 60 min centrifugation, and 60 min sonication on the protein content of the solutions before and after treatment was measured. For the non-centrifuged system, the calculated protein content was around 78.9 ± 2.0%, which is slightly less than the

manufacturer's specifications (84.2%). After 15- and 60-min centrifugation, the protein content decreased to 59.2 ± 1.3% and 51.9 ± 1.3%, respectively, indicating that some of the proteins in the original sample were insoluble. The sonicated sample had a protein content of 60.8 ± 6.0%. Emulsions prepared from pea proteins that had undergone overnight dissolution and then either 60 min centrifugation or sonication had bimodal particle size distributions and were unstable to creaming. However, the emulsions formed from pea proteins that had been centrifuged for 15-mins had monomodal distributions and were the most stable to creaming.

In summary, these preliminary experiments indicated that the best procedure to remove the insoluble pea proteins was to disperse them in water, incubate them overnight in a refrigerator, and then centrifuge them for 15 minutes.

Influence of emulsifier concentration on emulsion formation

In these experiments, we aimed to determine the optimum level of pea protein needed to form the emulsions. The influence of pea protein concentration on the size of the oil droplets in the vitamin-loaded flaxseed emulsions was therefore measured using fixed homogenization conditions. As anticipated, the mean particle diameter (d_{32}) initially became smaller as the pea protein level was increased, but then reached a plateau value. This initial decrease was because the smallest droplet size that could be created was limited by the amount of surface area that could be covered by the available pea protein. The later plateau value was because there was sufficient emulsifier present, but the homogenizer could not produce any smaller droplets at the operating conditions used. These experiments showed that about a 1.5:10 ratio of pea protein-to-oil was needed to create relatively fine droplets and that the smallest particle diameter that could be generated was around 0.34 μm at 2% w/w protein.

The surface load (Γ) of the pea protein was estimated from a plot of d_{32} versus the reciprocal of the emulsifier concentration as described elsewhere.[30] This approach is based on the following expression: $d_{32} = 6\Gamma\phi/C$, where ϕ is the volume fraction of the oil droplets, and C is the concentration of emulsifier adsorbed to the oil droplet surfaces. The value of Γ calculated using this approach was 15.6 mg m^{-3}, which is considerably higher than the value of around 1 to 2 mg m^{-3} reported for dairy proteins, such as whey protein and caseinate.[10] This suggests that the pea proteins formed a thicker interfacial layer at the droplet surfaces. This results is consistent with the fact that pea proteins are known to have relatively high molecular weights and a tendency to aggregate in aqueous solutions.[31]

Impact of pH on emulsion stability

During processing or digestion, food or beverage products containing nanoemulsion droplets may be subjected to alterations in their pH values. We therefore investigated the effects of pH on the zeta-potential (ζ), mean droplet diameter (d_{32}), and physical stability of the pea-protein stabilized emulsions. Only the results for flaxseed oil are shown here because the other systems behaved very similarly.

The ζ-potential of the pea protein-coated droplets went from cationic at low pH to anionic at high pH. The effective isoelectric point (pI) of the adsorbed pea proteins was around pH 4.2 (Figure 2a). This value agrees with earlier studies that have reported the pI of pea proteins to be between pH 4 to 5.[31-33] The d_{32} of the emulsions was relatively low under highly acidic conditions (pH 2), as well as under basic conditions (pH 7 to 9), but was relatively high near the pI of the protein-coated droplets (Figure 2b). Moreover, no phase separation was observed visually at pH 2, 3, 7, 8, and 9, but considerable creaming was observed at pH 4, 5, and 6 (Figure 2b).

These effects are related to changes in the electrostatic forces acting amongst the protein-coated droplets when the pH is changed. At relatively low and high pH values, there is a high ζ-potential and strong electrostatic repulsion. Conversely, at intermediate pH values, around the isoelectric point, the protein-coated droplets only have a relatively weak electrostatic repulsion. In fact, there may even be an electrostatic attraction between the droplets because of surface heterogeneity, *i.e.,* positive regions on one protein bind to negative regions on another. Consequently, the van der Waals, hydrophobic, and electrostatic attraction amongst the droplets is sufficiently intense to overcome the weak electrostatic repulsion, causing the oil droplets to aggregate. Similar results have also been reported for pea protein-coated oil droplets in other studies.[34, 35]

Impact of carrier oil type on emulsion formation

It is well known that carrier oil type can impact the production and stability of emulsions, as well as the bioaccessibility of hydrophobic bioactive substances. We therefore examined the influence of three carrier oils on emulsion formation and performance: corn oil (plant-based monounsaturated); flaxseed oil (plant-based polyunsaturated); and, fish oil (animal-based polyunsaturated).

Initially, the impact of the kind of carrier oil used on the particle size characteristics of the vitamin-fortified nanoemulsions was measured when they were produced using standardized homogenization conditions: pressure, passes, and composition. Previous studies

suggest that lipid digestion and bioactive bioaccessibility increase as the lipid droplet size decreases,[36, 37] and so it is advantageous to create emulsions with small droplet sizes. Emulsions containing relatively small oil droplets could be generated using all three carrier oils ($200 < d_{32} < 550$ nm) and they had monomodal distributions (Figure 3). These results agree with previous research, which also reported that relatively small droplets could be produced using pea proteins with a various kinds of oils, including fish oil [34], soybean oil [38], and sunflower oil [35]. The relatively small size of the droplets in all of the emulsions prepared in this study should be suitable for creating products with good physical stability, rapid lipid digestion, and high vitamin bioaccessibility.

Interestingly, the emulsions formed with corn oil had smaller particle diameters, than those formed with either flaxseed oil or fish oil (Figure 3). This difference in particle size may be due to the fact that the oils have different compositions and physicochemical properties. The triacylglycerols in corn, flaxseed, and fish oil mainly contain long-chain fatty acids and are rich in unsaturated fatty acids. Fish oil contains high levels of eicosapentaenoic acid (EPA: C20:5n-3) and docosahexaenoic acid (DHA: C22:6n-3). Flaxseed oil is high in oleic acid (18:1n-9), linoleic acid (LA: C18:2n-6), and alpha-linolenic acid (ALA: C18:3n-3). Whereas corn oil is high in linoleic acid and oleic acid. There may also be different kinds of surface-active impurities in the oils that could influence emulsion formation or stability.

Impact of carrier oil type on GIT fate

The impact of the carrier oil used to formulate the vitamin-fortified emulsions on their gastrointestinal fate was established by utilizing a three-stage static *in vitro* GIT model. The vitamin D_3 (0.1 % w/w) was dissolved in fish oil, flaxseed oil, or corn oil prior to preparing the emulsions. The different emulsions were then sequentially incubated in the different stages of the simulated GIT. We hypothesized that the different kinds of carrier oil would alter the behavior of the oil droplets under gastrointestinal conditions. We therefore examined the influence of oil type on emulsion characteristics in the different regions of the GIT.

All emulsions exhibited fairly similar behavior throughout the simulated GIT. In terms of their surface potentials, a slight decrease of the net negative charge on the droplets occurred after they were incubated in the artificial saliva (Figure 4a), which has been attributed to electrostatic screening and mucin adsorption.[39, 40] The particle charge was close to zero for all the emulsions after exposure to the simulated gastric fluids, presumably because of charge neutralization effects caused by attachment of negatively-charged mucin to the surfaces of positively-charged protein-coated oil droplets under acidic stomach conditions.[5] In

the small intestine, the ζ-potential was again highly negative, which can be account for by the existence of an assortment of anionic components in the intestinal fluids after lipid digestion, such as bile acids, peptides, mucin, and free fatty acids.

The emulsions containing the different oil phases also showed fairly similar behavior in terms of their particle sizes and microstructures in the different regions of the GIT. Light scattering indicated that the particle size increased slightly when the emulsions moved into the mouth (Figure 4b), which was supported by the confocal microscopy experiments (Figure 5), suggesting that some particle aggregation had occurred. Previously, this effect has been linked to electrostatic screening by the counter-ions from the salts in the artificial saliva, as well as to bridging and depletion flocculation associated with the presence of mucin.[40, 41] In the stomach, light scattering indicated that there was a large increase in particle size, while the microscopy images clearly showed that flocculation had occurred, indicating that incubation in simulated gastric fluids induced widespread aggregation of the protein-coated oil droplets. This phenomenon is related to the decrease in electrostatic repulsive forces amongst the oil droplets caused by the alteration in pH and ionic strength, as well as due to bridging flocculation by the mucin.[42, 43] In the small intestine, there was still evidence of large particles in the samples in both the light scattering and confocal microscopy results. These particles probably arise from the digestion processes occurring in this phase of the GIT. In particular, lipid digestion will lead to the formation of partially digested oil droplets, micelles, vesicles, and calcium soaps.[44, 45] Visually, the emulsions initially had a homogeneous creamy white appearance. After being incubated in the mouth and stomach phases there was a white cream layer on top of the samples due to the rapid upward movement of the oil droplets when they flocculated. The creaming velocity is proportional to the particle size squared, so the greater particle size for the flocs would have led to more rapid creaming. At the end of the small intestine phase, the samples had a light brown turbid appearance, with no evidence of a cream or oil layer on their surfaces, which was indicative of full digestion of the lipid droplets. The slight brown color comes from the gastrointestinal components, such as bile salts.

Overall, these results indicated that carrier oil type did not have a larger impact on the gastrointestinal fate of the different vitamin-fortified emulsions.

Impact of carrier oil type on lipid digestion kinetics

The impact of the carrier oil used to formulate the vitamin-fortified emulsions on their lipid digestion kinetics was determined within the small intestine phase of the GIT model.

We hypothesized that the different types of carrier oil would be digested at different rates and extents, thereby impacting vitamin bioaccessibility.

The generation of FFAs by the emulsions within the small intestine was measured by automatic titration (Figure 6). The general FFA-time profiles were similar for all three carrier oils: most of the lipids were rapidly digested within the first 10 minutes, while the remainder were slowly digested over longer times. However, there were some differences between the samples. The corn oil emulsions were digested quicker during the initial stages and generated more FFAs by the end of the small intestine phase than the flaxseed or fish oil emulsions. These results suggest that the carrier oil comprising mainly of monounsaturated fatty acids (MUFAs: corn oil) was digested more effectively than the carrier oils containing polyunsaturated fatty acids (PUFAs: flaxseed and fish oils). There may be a number of physicochemical processes that would account for this phenomenon. The digestive enzymes adsorbed to the droplet surfaces may not have been able to cleave the ester bonds in the PUFAs as easily as in the MUFAs because of steric hindrance effects. The multiple double bonds in the PUFAs mean that the fatty acid chains adopt a highly bent conformation, which may restrict the access of the lipase. Moreover, the formation of vesicles and micelles at the surfaces of the fat droplets during digestion may have been impacted by the molecular conformation of the fatty acids.[46] Normally, the long chain FFAs produced during the digestion of emulsified lipids have to be removed from the oil droplet surfaces, either by being solubilized by bile salts or by producing insoluble complexes with calcium.[45] These processes may be influenced by the precise molecular structure of the fatty acids present at the oil-water interface.

In summary, the emulsions formulated using MUFAs (corn oil) were digested more rapidly and fully than those formulated using PUFAs (flaxseed and fish oil).

Influence of carrier oil on vitamin D_3 bioaccessibility

Finally, the influence of carrier oil type on the bioaccessibility of the vitamins encapsulated in the different nanoemulsions was determined. We hypothesized that because the carrier oils were digested to different rates and extents they would lead to different bioaccessibilities. This turned out to be the case. The vitamin bioaccessibility was significantly ($p < 0.05$) higher for the corn oil emulsion than for the flaxseed or fish oil emulsions (Figure 7). Indeed, the bioaccessibility was around 78% for the MUFAs system, but only around 43% for the two PUFAs systems. This result shows that carrier oil type can have a pronounced impact on the bioaccessibility of this important micronutrient. There may

be numerous reasons for this phenomenon. First, some of the vitamins may have remained trapped inside the PUFA-rich droplets because they were not completely digested within the small intestine (Figure 6). As a result, they did not become part of the mixed micelle fraction. Second, the hydrophobic domains in the mixed micelles may have been smaller for PUFAs than for MUFAs because of the extensive kinking of the fatty acid chains. As a result, the PUFA-mixed micelles had a lower solubilization capacity than the MUFA-ones.

Various other researchers have also found that the nature of the carrier oil used to deliver highly hydrophobic bioactive substances has a major impact on their bioaccessibility. The bioaccessibility of both pro-vitamin A (β-carotene) and vitamin E (α-tocophernol) were reported to be higher when corn oil is used as a carrier oil rather than medium chain triglycerides (MCT).[47-49] For emulsions stabilized by another natural surfactant (quillaja saponins), the bioaccessibility of vitamin D_3 decreased in the following order: corn oil ≈ fish oil > orange oil > mineral oil > MCT.[27] Again, these effects have been attributed to differences in the extent of digestion of the carrier oil and the nature of the mixed micelles they form.

Conclusion

The study investigated the influence of carrier oil type on the production and performance of nanoemulsion-based vitamin D_3 delivery systems. These delivery systems were formulated using a natural emulsifier (pea protein) because of the growing trend in the food industry towards more plant-based foods. The research showed that the digestion and bioaccessibility characteristics of the vitamin-fortified emulsions was dependent on carrier oil type. In particular, the emulsions formulated using monounsaturated fatty acids (corn oil) were digested more rapidly and fully than those formulated using polyunsaturated fatty acids (flaxseed and fish oil). Moreover, vitamin bioaccessibility was appreciably higher in MUFA-emulsions than PUFA-emulsions. Differences in the nature of the fatty acid chains in the carrier oils may account for these effects. The PUFAs have highly kinked chains that may have inhibited the ability of the lipase to access the ester bonds and/or interfered with the formation of mixed micelles at the lipid droplet surfaces during digestion. These results may be beneficial for formulating more efficacious delivery systems for vitamin D. In future, it will be critical to establish whether the nanoemulsions developed in this study can be utilized in commercial products without negatively impacting their physicochemical or organoleptic properties, as well as to perform *in vivo* studies to confirm the influence of the nature of the carrier oil on vitamin bioaccessibility. Moreover, it would be of interest to carry out studies

in the future to locate the precise location and orientation of the vitamin D within the protein-coated lipid droplets, *e.g.,* is it located at the droplet surfaces or within their interior. This type of molecular information would be useful for understanding and controlling the chemical stability and bioavailability of the vitamins within emulsion-based delivery systems. Knowledge of the location of the vitamin molecules within emulsions may be obtained using analytical techniques such as electron paramagnetic resonance spectroscopy (EPR) or diffusion ordered NMR spectroscopy (DOSY).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figures

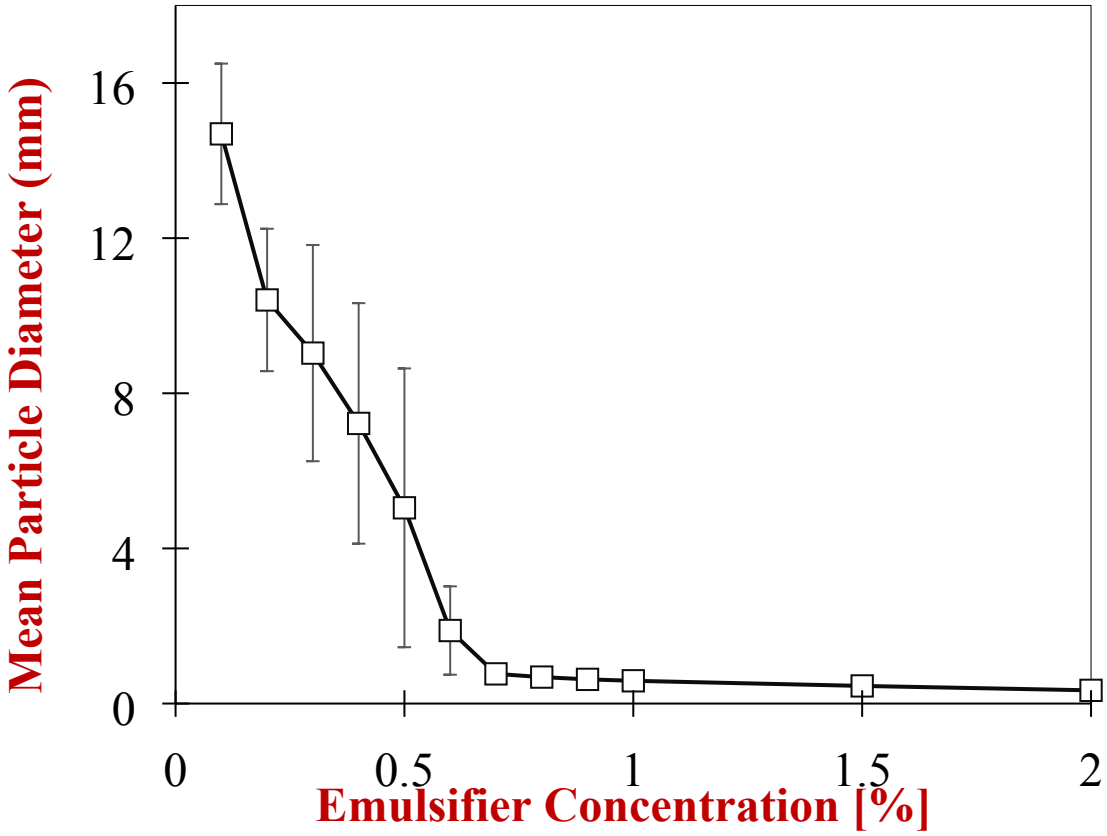
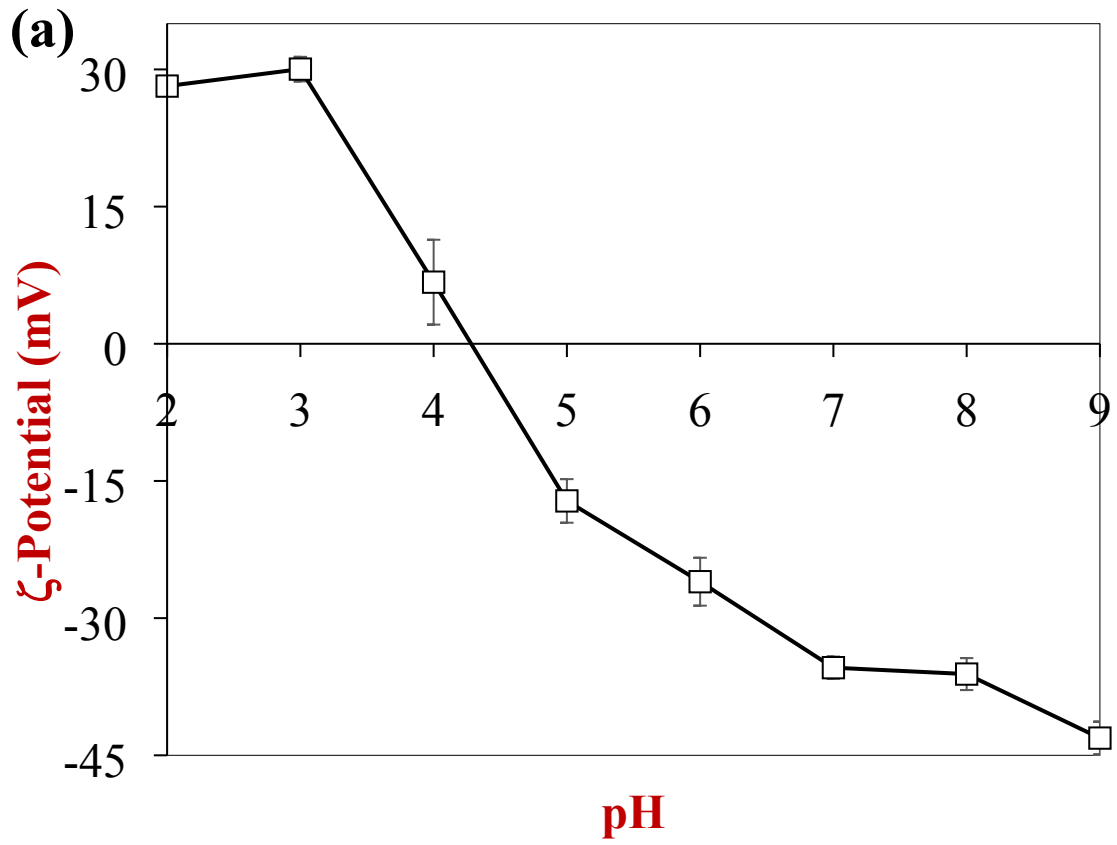


Figure 1. Impact of pea protein concentration on the mean droplet diameter (d_{32}) of oil-in-water emulsions after homogenization. The emulsions contained 90 % w/w aqueous phase and 10 % w/w oil phase (9.9 % w/w flaxseed oil + 0.1 % w/w vitamin D_3) at pH 7.


(a)
30
15
0
-15
-30
-45
2
3
4
5
6
7
8
9
ζ-Potential (mV)
pH

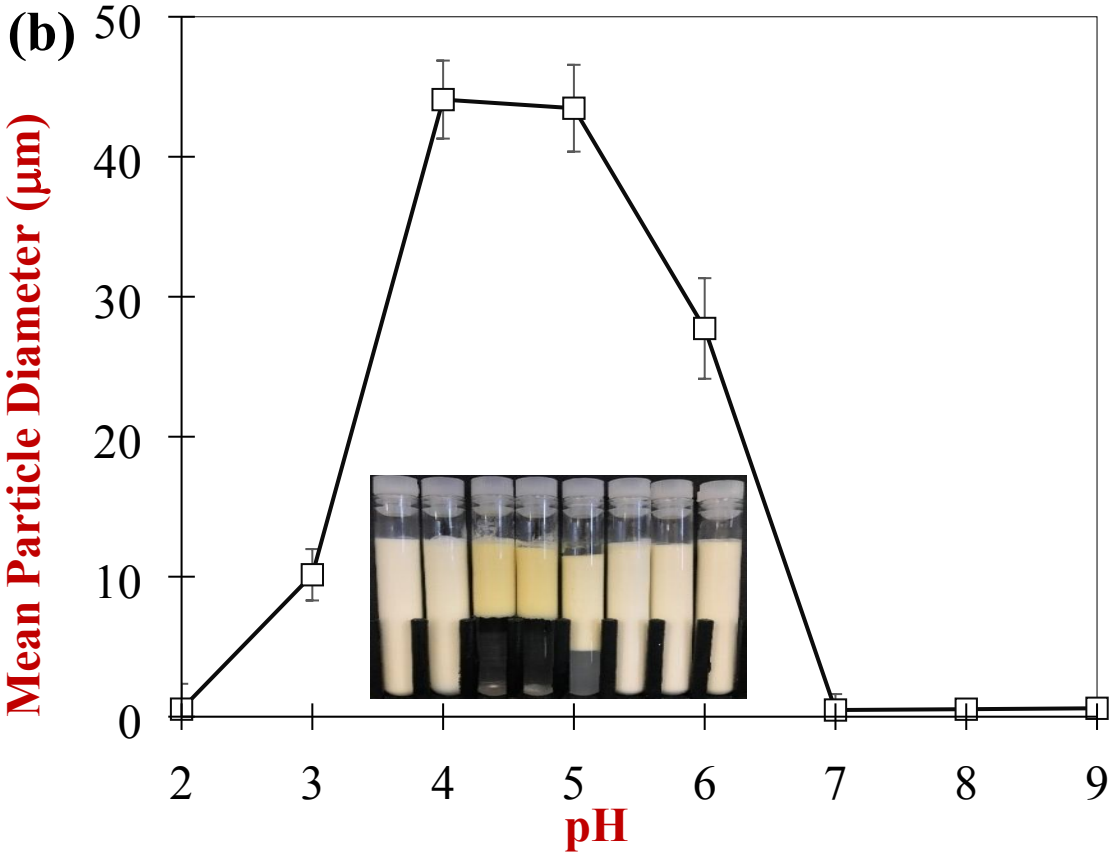

(b)
50
40
30
20
10
0
2
3
4
5
6
7
8
9
Mean Particle Diameter (μm)
pH

Figure 2. Zeta-potential (a), mean particle diameter (d_{32}) and appearance (b) of vitamin-fortified emulsions. The initial emulsions were prepared by homogenizing 90 % w/w aqueous phase, 9.9 % w/w flaxseed oil, and 0.1 % w/w vitamin D_3.

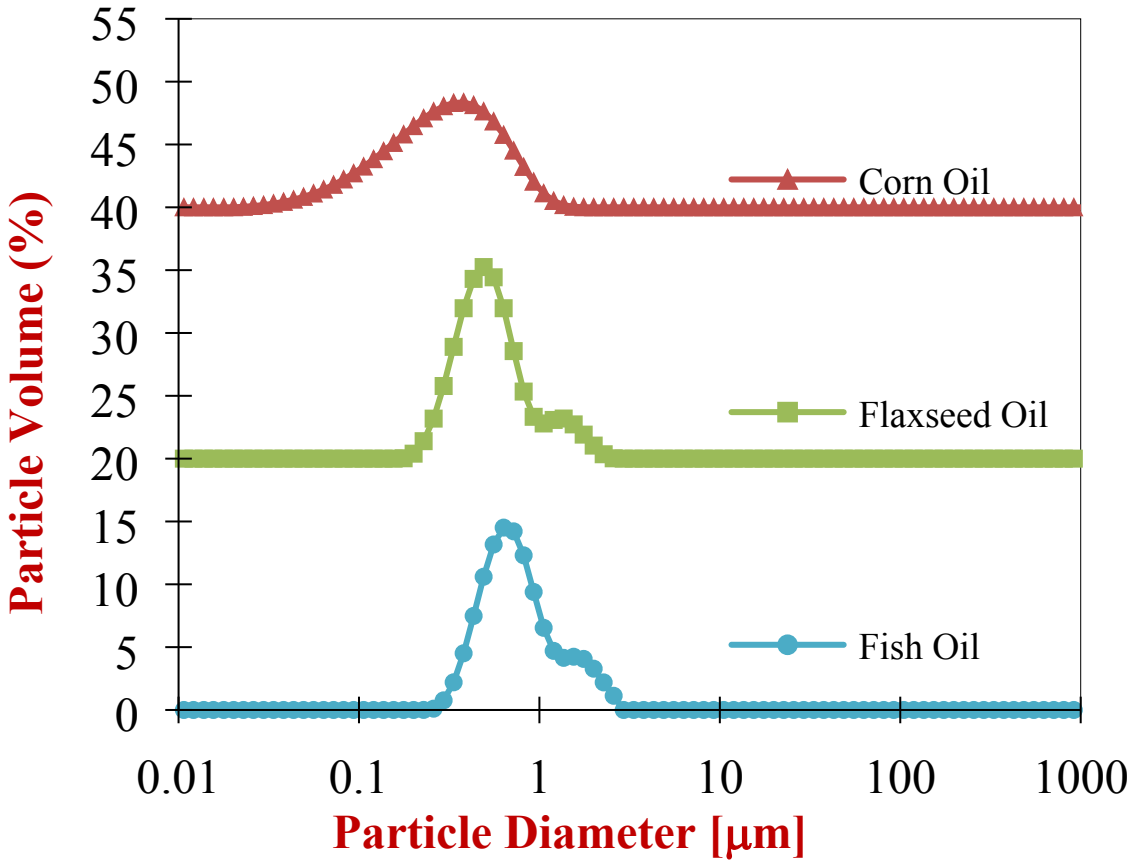
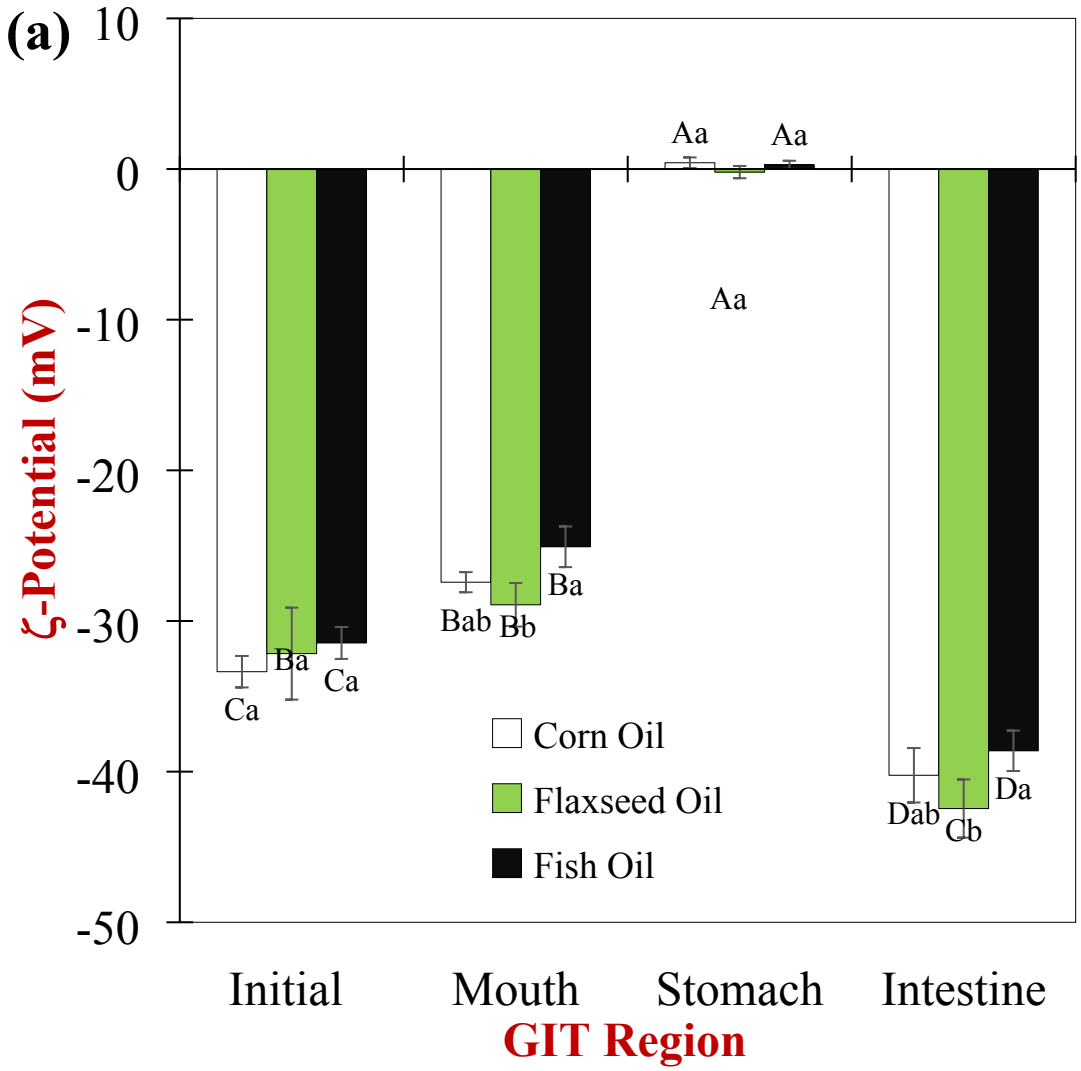


Figure 3. Impact of oil type on the initial mean particle diameter (d_{32}) and particle size distribution of vitamin-fortified pea-protein stabilized emulsions. The initial emulsions were prepared by homogenizing 90 % w/w aqueous phase, 9.9 % w/w flaxseed oil, and 0.1 % w/w vitamin D_3.


(a)
10
0
-10
-20
-30
-40
-50
ζ-Potential (mV)
Aa
Aa
Aa
Ca
Ba
Ca
Bab
Bb
Ba
Dab
Cb
Da
Corn Oil
Flaxseed Oil
Fish Oil
Initial
Mouth
Stomach
Intestine
GIT Region

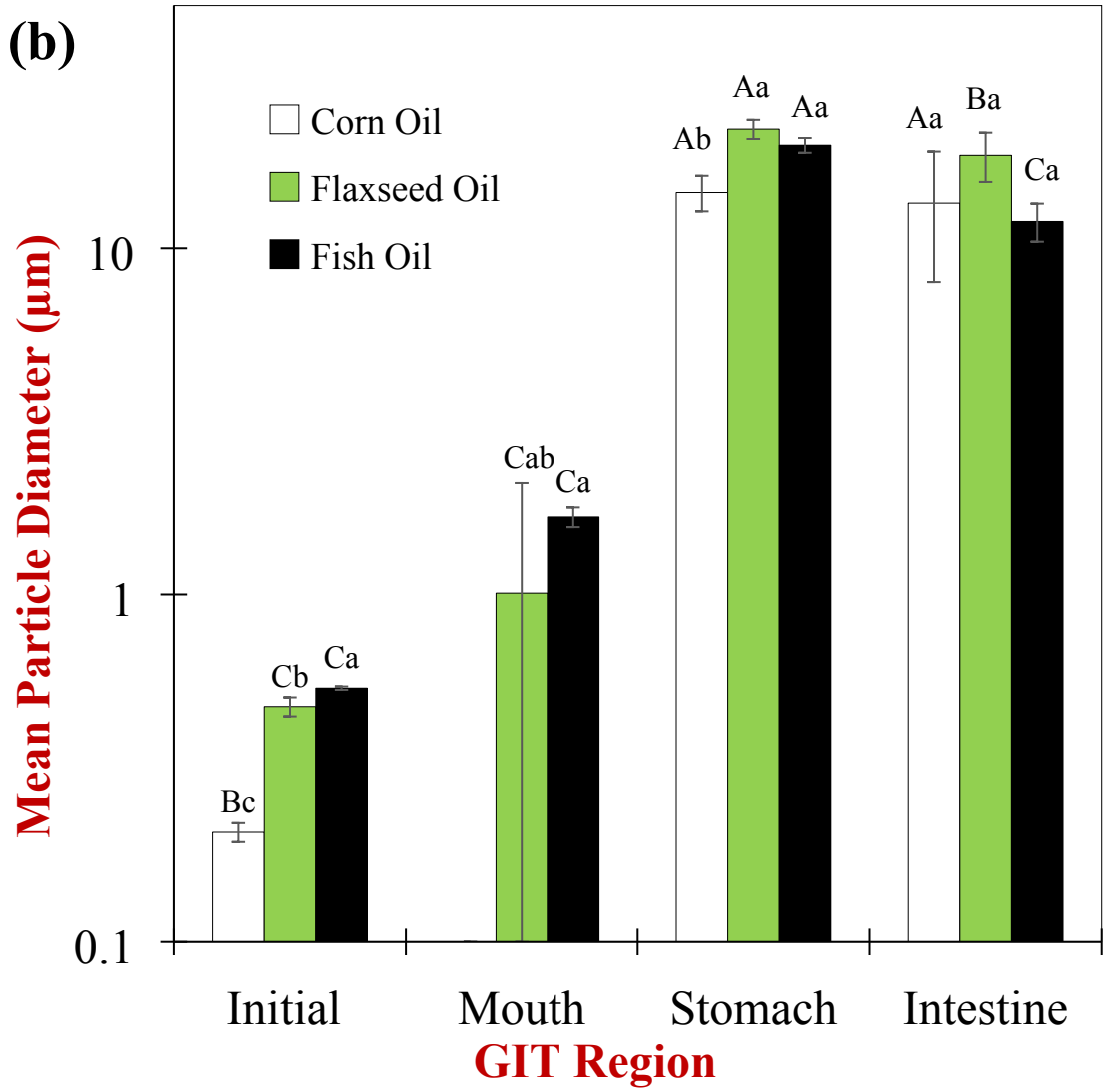


Figure 4. Impact of carrier oil type on the zeta-potential (a) and mean particle diameter (b) of vitamin D_3-fortified emulsions passed through a simulated GIT.

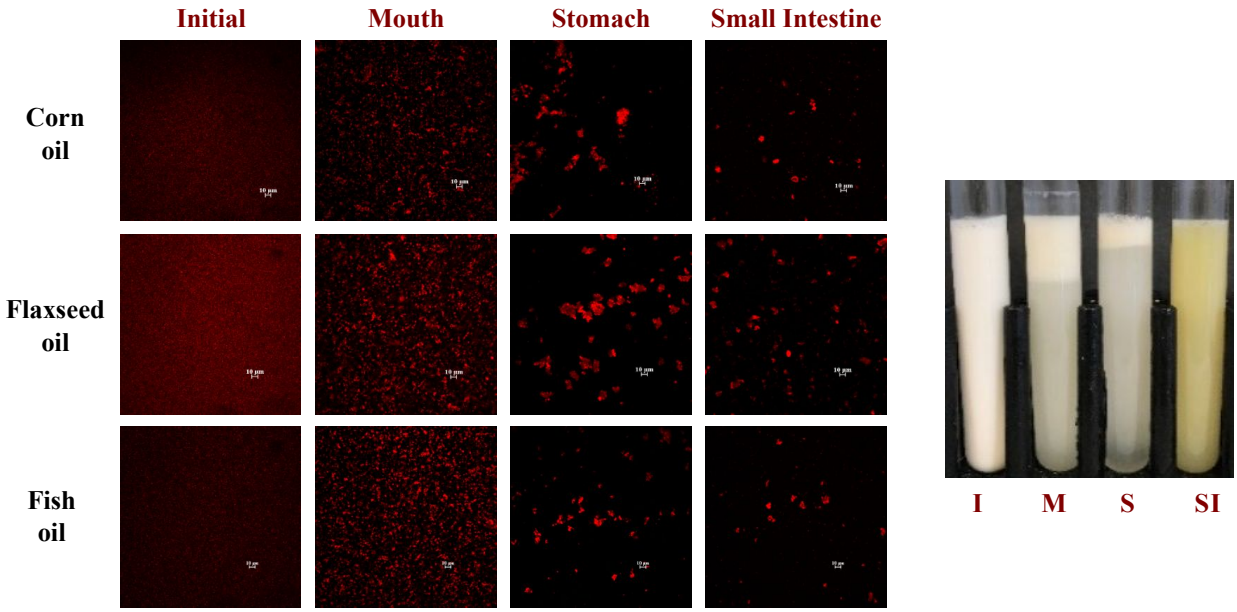


Figure 5. Confocal microscopy images of pea-protein stabilized emulsions formulated with different carrier oil types. The initial emulsions were subjected to a simulated GIT consisting of mouth, stomach, and small intestine phases. The photograph shows the visual appearance of the emulsions after exposure to different GIT phases (all emulsions looked fairly similar).

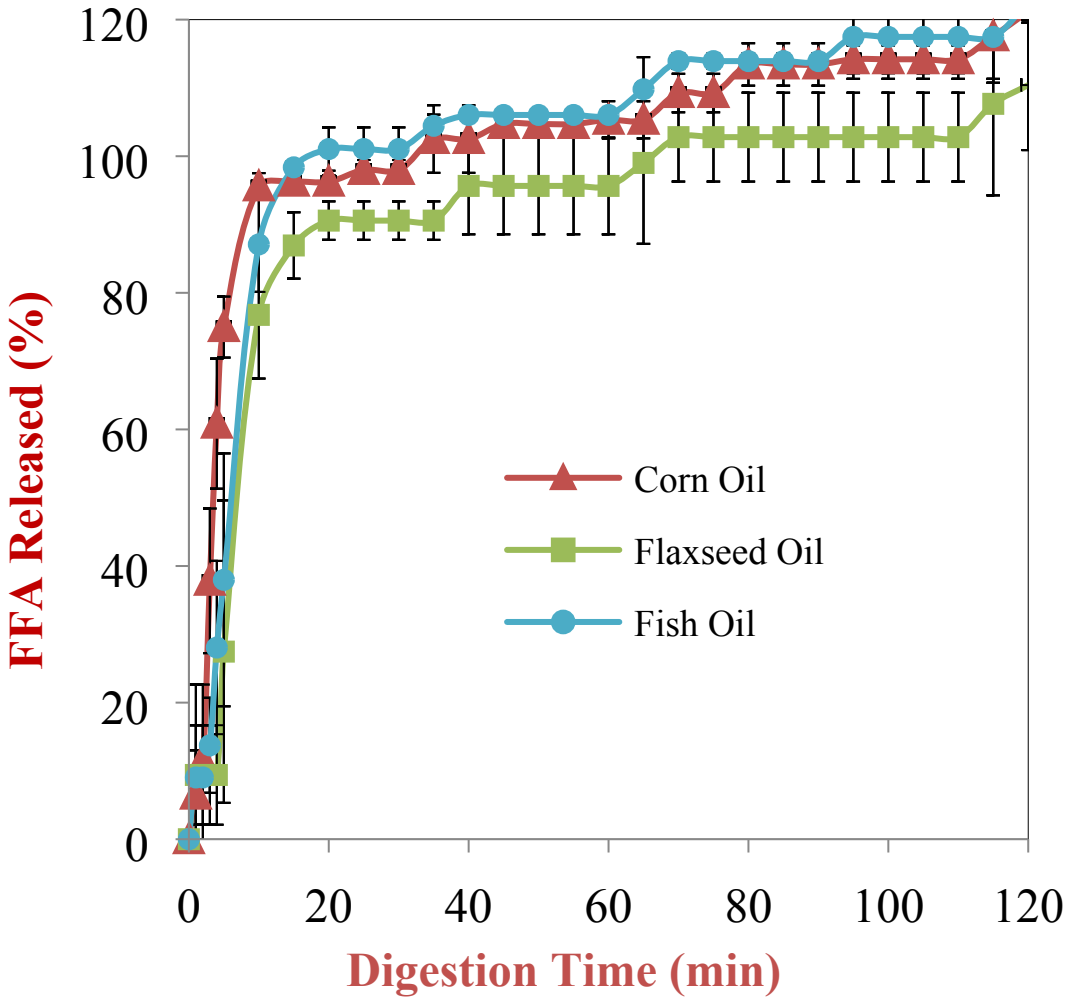


Figure 6. Influence of carrier oil type on the release of free fatty acids (FFA) from pea-protein stabilized emulsions during incubation in a simulated small intestine phase.

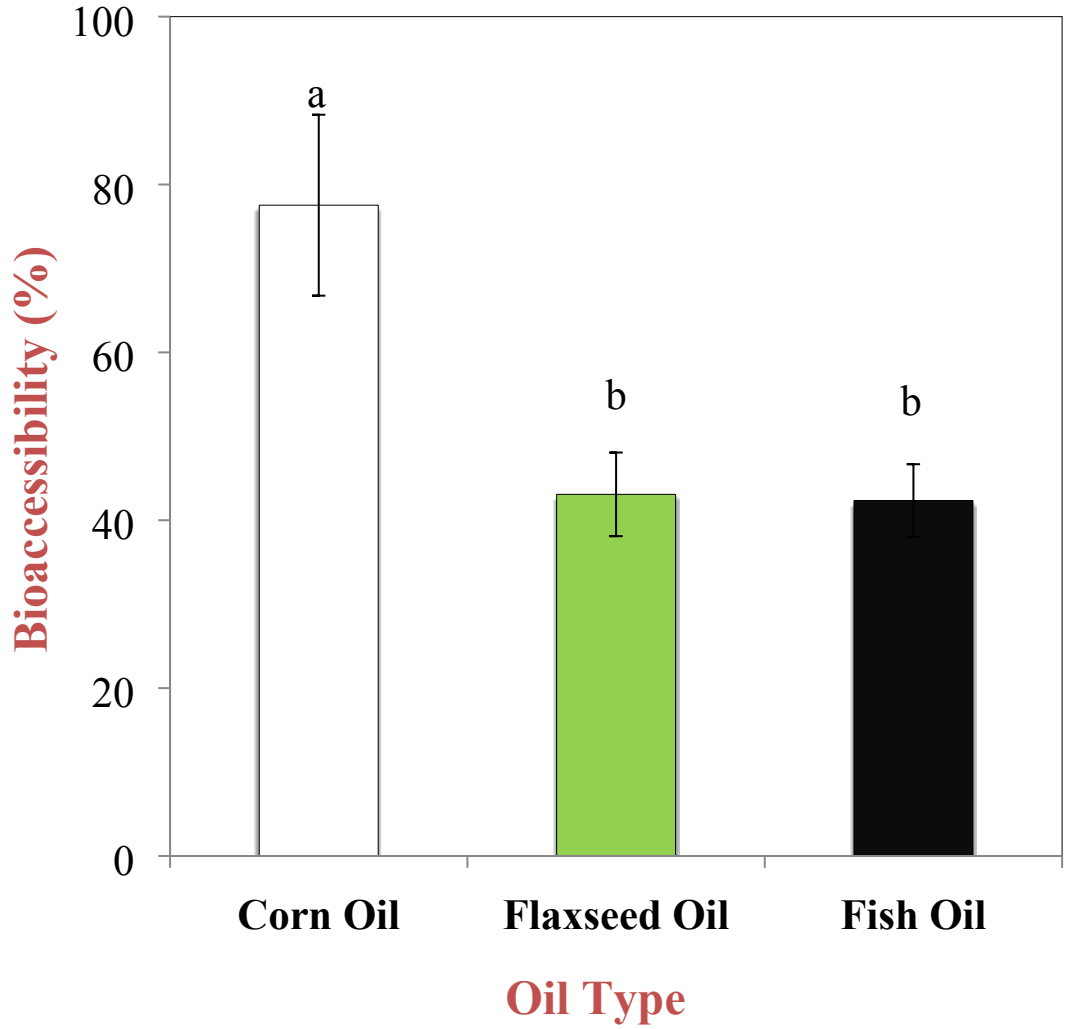


Figure 7. Influence of carrier oil type on the bioaccessibility of vitamin D_3 after pea-protein stabilized emulsions were exposed to a simulated GIT tract.

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Graphical Abstract