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Release and Bioaccessibility of β-Carotene from Fortified Almond Butter during in Vitro Digestion

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ABSTRACT: The objective of this study was to determine the release and bioaccessibility of β-carotene from fortified almond butter using in vitro digestion models. Two types of fortifiers were investigated: β-carotene oil (oil) and whey protein isolate (WPI)-alginate-chitosan capsules containing β-carotene oil (capsule). Shaking water bath and Human Gastric Simulator (HGS) digestion models assessed the impact of gastric peristalsis on the release of β-carotene. Bioaccessibility of β-carotene was measured as percent recovered from the micelle fraction. There was greater release of β-carotene from oil fortified almond butter in the HGS model (87.1%) due to peristalsis than the shaking water bath model (51.0%). More β-carotene was released from capsule fortified almond butter during intestinal digestion. However, more β-carotene was recovered from the micelle fraction of oil fortified almond butter. These results suggest that a WPI-alginate-chitosan capsule coating may inhibit the bioaccessibility of β-carotene from fortified almond butter.

KEYWORDS: *encapsulation, β-carotene, alginate, chitosan, in vitro digestion, almond butter*

■ INTRODUCTION

Carotenoids are lipid soluble phytonutrients that are responsible for the yellow, orange, and red colors in fruits and vegetables.[1] Certain carotenoids, such as β-carotene and β-cryptoxanthin, have been a subject of interest for research due to their provitamin A activity and antioxidant properties.[2,3] However, their bioactivity is limited by their stability against oxidative degradation during storage and bioaccessibility from plant food matrix during digestion.[4] Furthermore, carotenoid absorption and bioavailability vary widely (>100-fold) in healthy individuals, a problem that impacts fortification programs using carotenoids to improve vitamin A status.[5,6]

Microencapsulation of carotenoids is a method to standardize and perhaps increase bioaccessibility by controlled release during digestion. Bioaccessibility is calculated as the fraction of carotenoids that is released from a food matrix and incorporated into mixed micelles that can be absorbed in the small intestine.[7] Several research groups have successfully microencapsulated carotenoids in polymer matrixes and evaluated their shelf stability.[8−12] However, this research has yet to assess the release of carotenoids that have been microencapsulated from a food matrix during digestion. Shen et al.[13] compared the release of polyunsaturated fatty acids from microencapsulated tuna oil that had been incorporated into various food matrixes (orange juice, yogurt, and cereal bar) using an in vitro digestion model and found that the structure (solid) and composition (fiber) of a food matrix can inhibit release of encapsulated lipids. In addition, there is a lack of research that examines the bioaccessibility of carotenoids that have been used to fortify foods in a nonencapsulated as compared to an encapsulated form. Matalanis et al.[14] assessed the rate of lipid digestion of hydrogel (pectin−sodium caseinate) encapsulated and sodium caseinate stabilized oil in water emulsions using an in vitro pH static digestion model. Encapsulation did not cause a significant difference in the rate of lipid digestion. However, that study did not measure the amount of lipid that had been incorporated into mixed micelles. Carotenoids must be incorporated into mixed micelles to be bioaccessible.

In vitro digestion models are becoming increasingly popular for predicting the bioaccessibility of carotenoids, because their results provide qualitative insights into factors that influence carotenoid bioavailability. In vitro digestion models can be classified as static or dynamic models. Static models do not attempt to mimic the mechanical and physiological processes that occur in vivo, such as pH change and peristalsis, whereas dynamic models attempt to mimic these processes.[15] The most widely used model to evaluate bioaccessibility of carotenoids is a static in vitro digestion coupled with a Caco-2 cell absorption model developed by Garrett et al.[7] Similar to the Garrett in vitro digestion model, most models used in carotenoid bioaccessibility studies are static models that do not examine the release of carotenoids during gastric digestion and do not mimic the mixing patterns and forces that occur in the stomach due to peristalsis. The Human Gastric Simulator (HGS), developed by Kong and Singh,[16] is a dynamic gastric digestion model that is designed to mimic the peristaltic movements that occur in the stomach. This model may provide a more accurate simulation of the food disintegration and carotenoid release that occurs in vivo than previously tested static digestion models.

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The objective of this study was to determine the effect of whey protein isolate (WPI)-alginate-chitosan encapsulation on the release of β-carotene from fortified almond butter during in vitro digestion using a static (shaking water bath) and a dynamic (HGS) model.

■ MATERIALS AND METHODS

Materials. Low viscosity alginic acid, low molecular weight chitosan, calcium chloride dehydrate, porcine pancreatin (CAS: 8049-47-6), porcine mucin, and porcine bile extract were purchased from Sigma-Aldrich (St. Louis, MO). WPI was provided by Hilmar Ingredients (Hilmar, CA). Lucarotin 30M, a 30% β-carotene in corn oil dispersion, was provided by BASF (Ludwigshafen, Germany). Homestyle Creamy Almond Butter was provided by Blue Diamond (Sacramento, CA). The macronutrient composition of Blue Diamond Homestyle Creamy Almond Butter is as follows: 53% lipid, 22% protein, 19% carbohydrates (13% dietary fiber; 6% sugar), and 6% water, ash, and other. α-Amylase (CAS: 9000-90-2, ~165 U/mg) was purchased from MP Biomedicals (Solon, OH). Lipase A (CAS: 9001-62-1, >12 U/mg) was donated by Amano Enzymes (Japan). Pepsin (CAS: 9001-75-6, >250 U/mg) and analytical grade salts and chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

Preparation and Characterization of Encapsulated β-Carotene Oil. Alginate and chitosan were chosen as encapsulating materials because the coating has previously exhibited enteric (intestine) release properties.[17−19] Because absorption of β-carotene takes place at the beginning of the small intestine,[20] enteric release should promote absorption. WPI was chosen as an emulsifier for the β-carotene oil-in-water emulsion because it has been previously used to stabilize alginate-chitosan capsules.[21] WPI-alginate-chitosan capsules containing β-carotene oil were prepared using an emulsion-ionic gelation technique based on previously described methods[18,19,21] under nitrogen gas and yellow light to minimize oxidative degradation. A β-carotene/whey protein isolate (WPI) emulsion was prepared by mixing Lucarotin 30 M (7 g) and 1% WPI (63 g) with a hand-held homogenizer (Biohomogenizer, Fisher Scientific, Fair Lawn, NJ) on high for 3 min. To make the encapsulating medium, β-carotene/WPI emulsion (60 mL) was stirred with 2.5% sodium alginate (240 mL) for 30 min. The encapsulating medium was 2% sodium alginate, 0.18% WPI, and 0.6% β-carotene, pH 5.0.

The gelation medium was a 4% calcium chloride solution (1500 mL), pH 5.5. The encapsulating medium was loaded into an encapsulation system, comprised of six 60 mL syringes equipped with 27 gauge needles, and dropped into the continuously stirred gelation medium at a pressure of 100 kPa. The flow rate of the encapsulating medium was regulated by nitrogen gas.

After all of the encapsulating medium was dropped into the gelation medium and allowed to set for at least 30 min, 1% chitosan in 1% acetic acid, pH 5.0 (1500 mL), was added to the gelation medium. This changed the composition of the gelation medium to 2% calcium chloride and 0.5% chitosan, pH 5.0. The capsules were stirred in this gelation medium for an additional 30 min to allow the adhesion of the chitosan coating.

The capsules formed were drained into a mesh bag (297 μm) and rinsed with distilled water. These capsules were dried by mixing with rice (30 g), a desiccant , an air blast (2.5 m/s), and periodic manual shaking (every 10 min) in the mesh bag for 1 h. Capsules were separated from rice particles by sieving through an 850 μm sieve (Endecotts, London, UK) and stored under nitrogen in an amber bottle at refrigeration temperature (4 °C).

Particle size distribution of the dried capsules was measured by dry sieving. Median particle size was determined by fitting the cumulative weight undersize distribution to a log-normal distribution using Matlab (R2011b, Mathworks, Natick, MA). The median particle size yielded by this method was 762 ± 32 μm.

The surface morphology of the capsules was assessed using scanning electron microscopy (SEM). Dried WPI-alginate-chitosan capsules were placed on a small aluminum platform with double-sided carbon and observed in a Hitachi S-4100T FE-SEM (Japan) at 2 kV.

Preparation of Fortified Almond Butter. Almond butter fortification was targeted to 50% RDA for a 19+ male adult (5.4 mg of β-carotene)[22] per serving (32 g). Creamy almond butter was chosen as a food matrix because it is uniform, lipophilic, and rich in antioxidants, which can protect β-carotene from degradation during storage and digestion.

Oil Fortification. The method for fortifying almond butter with β-carotene oil was adapted from a study conducted by the United States Agency for International Development Peanut Collaborative Research Support Program.[23] One jar of Homestyle Creamy Almond Butter (340 g) was heated to 65 °C in a shaking water bath (YB-531, American Scientific Products, McGraw Park, IL). Approximately 0.2 g of Lucarotin 30 M was added to the jar to achieve the desired fortification level. The almond butter was hand mixed for 5 min, while holding at 65 °C. After being mixed, the almond butter was cooled in a 10 °C water bath for 2 h and then transferred to a refrigerator and stored for at least 48 h at 4 °C. A control almond butter sample was prepared by substitution of locally purchased corn oil for Lucarotin 30M.

Capsule Fortification. Almond butter fortified with encapsulated β-carotene oil was prepared on the day of use to match the fortification level of oil fortified almond butter, which was 1.8 mg capsules/g almond butter (±10%; Table 1).

Table 1. β-Carotene Contents of Almond Butters and WPI-Alginate-Chitosan Capsules[a]

	control almond butter (μg/g)	fortified almond butter (μg/g)	WPI-alginate-chitosan capsules (mg/g)
target β-carotene content	0	168.75	100
actual β-carotene content	0.477 ± 0.14	147.0 ± 32	82.9 ± 7.5

[a]Almond butter concentration is expressed as μg/g, and capsule concentration is expressed as mg/g. Error is standard deviation.

In Vitro Digestion Model for Almond Butter. *Development of High Lipid Food Digestion Model.* An in vitro digestion model for evaluating the bioaccessibility of nutrients in a high lipid system was developed to evaluate the release of nutrients from a high lipid food matrix during gastric digestion. The simulated saliva and gastric juice used for this model were adapted from Kong and Singh[24] with modifications to more accurately simulate digestion of a high lipid food matrix. The duration of the saliva phase was adjusted to 30 s to reflect in vivo measurement of swallowing time for peanut butter.[25] Lipase A, derived from *Aspergillus niger*, was added to the simulated gastric juice to mimic human gastric lipase, which is commercially unavailable. Lipase A has been used as a gastric lipase substitute in a previous study.[26] The activity of the lipase was matched to that of the fasted gastric lipase activity recorded in a human clinical study, 40 U/mL.[27] During the gastric phase, pH was adjusted three times over the course of the 2 h digestion, beginning at pH 4 and then adjusting to pH 3 and pH 2, to reflect pH changes that occur during the fed state of digestion that have been measured in vivo.[28] An intestinal phase adapted from Wright et al.[29] and chosen for optimum micellarization of β-carotene was performed to determine bioaccessibility of β-carotene. The ratios of food to digestive juices used in the model are adapted from the RIVM (National Institute of Public Health and the Environment, The Netherlands) fed state digestion model,[30] as follows: 1.5 (food intake):1 (saliva):2 (gastric juice):2 (pancreatic juice):1 (bile). This ratio is based on the amount of digestive juices secreted on a daily basis by a typical healthy human adult. Stock concentrations of simulated gastric and intestinal juices were adjusted for dilution factors, because they were added to preexisting digestion mixtures. The composition of the simulated digestive solutions is shown in Table 2.

Table 2. Composition of the Digestive Solutions for High Lipid in Vitro Digestion Model

simulated saliva	stock (mg/mL)	final (mg/mL)
α-amylase	2	2
mucin	1	1
NaCl	0.117	0.117
KCl	0.149	0.149
$NaHCO_3$	2.1	2.1
simulated gastric juice	stock (mg/mL)	final (mg/mL)
pepsin	1.5	1
lipase A	5	3.33
mucin	2.25	1.5
NaCl	13.2	8.78
simulated intestinal juice	stock (mg/mL)	final (mg/mL)
pancreatin	4.8	2.4
bile extract	20	10
$NaHCO_3$	33.6	16.8

Shaking Water Bath Model. Digestion was performed in a shaking water bath (YB-531, American Scientific Products, McGraw Park, IL) set at 37 °C and shaking at 120 rpm to mimic physiological conditions. All simulated digestive juices were warmed to 37 °C before being added to the simulated digestion to activate enzymes. Shaking water bath digestions were conducted in 50 mL amber centrifuge tubes sealed under nitrogen gas to minimize oxidative degradation.

Aliquots of almond butter (5 g) were weighed into 50 mL amber centrifuge tubes. Simulated saliva (3.33 mL, pH 7.0) was added to almond butter, vortexed for 5 s, and then incubated in the shaking water bath for 30 s. Next, simulated gastric juice (6.66 mL) was added to this mixture, the pH was adjusted to 4.0 with 6 M HCl, and the sample was incubated for 40 min. The pH then was adjusted to 3.0 with 6 M HCl, and the sample was incubated for 40 min. Next, pH was adjusted to 2.0 with 6 M HCl, and the sample was incubated for 40 min. Simulated intestinal juice (10 mL) then was added to the mixture, the pH was adjusted to 6.5 with 5 M NaOH, and the sample was incubated for 2 h. Separate samples were prepared for each of the simulated stages of digestion (saliva, gastric pH 4, gastric pH 3, gastric pH 2, intestine). When the stage of digestion that was to be evaluated for a particular sample was completed, the sample was removed and stored on ice until analysis on the same day.

Human Gastric Simulator (HGS) Model. To evaluate the impact of simulated gastric peristaltic contractions on the release and bioaccessibility of β-carotene in vitro, the previously described shaking water bath model was adapted to incorporate the Human Gastric Simulator (HGS).[16] The gastric secretion and emptying mechanisms of the HGS were not used for this method. A plastic liner (Animal Reproduction Systems, Chino, CA) was placed into the stomach chamber to ease the removal of the simulated digesta from the HGS. The heating mechanism of the HGS, which is normally a broad spectrum light source, was replaced with a red heating lamp to minimize UV exposure, and the stomach chamber was flushed with a gentle stream of nitrogen gas throughout digestion to minimize oxidative degradation. In addition, the digestion method was scaled up 10 times to fulfill the minimum volume requirements of the HGS.

Almond butter (50 g) was weighed into a 250 mL glass beaker. Simulated saliva (33.33 mL, pH 7.0) was added to almond butter, and then incubated at 37 °C for 30 s while mixing with a spatula to simulate oral processing. Next, simulated gastric juice (66.66 mL) was added to this mixture, and the pH was adjusted to 4.0 with 6 M HCl, while being mixed on a stir plate. The contents of the beaker were added to the stomach chamber of the HGS, which was preheated to 37 °C. The mixture was then digested in the HGS for 2 h, with pH adjustments to pH 3.0 after 40 min and pH 2.0 after 80 min with 6 M HCl. HCl was mixed with the digesta via the peristaltic contractions of the HGS. During pH adjustments, the temperature of the stomach was maintained by the use of a portable space heater (HFHVP3, Holmes, Boca Raton, FL). After gastric digestion, the plastic liner containing digested almond butter was removed from the HGS, and its contents were emptied into a 500 mL amber glass bottle. Simulated intestinal juice (~100 mL) was added to the sample, the pH was adjusted to 6.5 with 5 M NaOH, and it was incubated in the shaking water bath at 37 °C and 120 rpm for 2 h. For a single simulated digestion, samples (~3 mL) were removed at each stage of digestion (saliva, gastric pH 4, gastric pH 3, gastric pH 2, intestine) and stored on ice until analysis on the same day.

Characterization of Almond Butter. *pH.* The pH of almond butter and digestion samples was measured using an IQ150 Hand-held pH/mV/Temperature Meter (IQ Scientific Instruments, Carlsbad, CA). pH measurements were taken at each stage of digestion, before and after pH adjustments.

Viscosity. The apparent viscosity of almond butter and digestion samples was measured using a Rheostress 1 (Thermo Haake, Karlsruhe, Germany) with a geometry of parallel plates (P35 Ti L) of diameter 35 mm. The gap was set at 3 mm for almond butter and saliva and 1 mm for gastric and intestine samples. Samples were placed on the rheometer, equilibrated to a temperature of 37 °C for 2 min, and then the shear stress was measured from 0.001 to 2 s^{-1}.

Extraction of β-Carotene from WPI-Alginate-Chitosan Capsules. β-Carotene was extracted from WPI-alginate-chitosan capsules by a dissolution and solvent extraction method. Approximately 1 mg of capsules was weighed and combined with 10 mL of US Pharmacopeia simulated intestinal juice (SIJ: 10 g/L pancreatin and 6.8 g/L potassium phosphate, pH 7.5) in a 15 mL centrifuge tube. This mixture was sealed under nitrogen and incubated for 2 h in a shaking water bath (YB-531, American Scientific Products, McGraw Park, IL) set at 37 °C with shaking at 120 rpm to completely dissolve the capsules. Dissolved capsules were extracted three times with hexane (2 mL). The hexane layer was separated by centrifugation at 2500 rpm for 10 min at 4 °C (RC-5, DuPont Sorvall Instruments, Wilmington, DE) for all extractions. The hexane extracts were combined, brought up to a volume of 6 mL, and then diluted 30-fold with hexane.

Extraction of β-Carotene from Oil Fortified Almond Butter. Almond butter fortified with β-carotene oil was extracted by a saponification and solvent extraction technique adapted from previous methods.[23,31] Technical grade ethanol containing 0.1% BHT (6 mL) and 35% percent potassium hydroxide in water (240 μL) were added to almond butter (1 g), and this mixture was tightly capped, vortexed for 20 s, and then saponified at room temperature for 30 min while continuously mixing on a test tube shaker (Multitube VX-2500, VWR, Bridgeport, NJ). After saponification was completed, deionized water (3 mL) was added to the sample to stop the reaction. Saponified samples were extracted three times with hexane (3 mL). The hexane extracts were combined and brought up to a volume of 10 mL with hexane.

Extraction of β-Carotene from Digesta and Micelle Fraction. Aliquots (~2 g) of simulated digestion samples were placed into Beckman Ultra Clear tubes and centrifuged at 36 000 rpm at 4 °C for 10 min (Beckman Optima TL100, Beckman Coulter, Palo Alto, CA). This centrifugation protocol was adapted from a previous method[32] and adjusted to account for differences in rotor size. For all simulated digestion samples, the upper oil and aqueous fractions were collected and characterized as digesta. A duplicate of the intestinal samples was centrifuged from which only the aqueous fraction was collected using a needle and syringe. A portion of the aqueous fraction was filtered through a 0.22 μm nylon syringe (Millipore, Bedford, MA) to isolate the micelle fraction.[7]

The extraction of β-carotene from digesta was adapted from Bengtsson et al.[33] An aliquot (0.1−0.6 g) of digesta was extracted using 3 mL of hexane/acetone/ethanol (50:25:25) containing 0.1% (w/v) BHT. The upper hexane layer collected from the sample and the digesta was extracted once more using 1.0 mL of hexane. The hexane extracts collected were brought up to a volume of 3 mL with hexane.

Quantitation of β-Carotene from Extractions. The β-carotene content of hexane extracted samples was determined by spectrophotometry. The absorbance of β-carotene hexane extracts was read at 451 nm in 1 cm quartz cuvettes on a Spectronic Genesys 5 (Thermo

Electron Corp., Madison, WI). Concentration of β-carotene was calculated by the following equation based on the Beer–Lambert law:

$$\text{beta carotene}\left(\frac{\mu g}{g}\right) = \frac{A \times \text{volume (mL)} \times 10^4}{A_{1cm}^{1\%} \times \text{sample weight (g)}}$$

where A is absorbance at 451 nm, volume (mL) is volume of hexane extract in milliliters, $A_{1cm}^{1\%}$ is mass absorption coefficient for β-carotene in hexane at 451 nm, and $A_{1cm}^{1\%}$ = 2598 is the mass absorption coefficient calculated from the molar absorption coefficient published in the CRC Handbook of Chromatography.[34]

Statistical Analysis. Shaking water bath digestions were conducted in quadruplicate, and HGS digestions were conducted in triplicate. Results are expressed as mean ± standard deviation. A three factor analysis of variance (ANOVA) was conducted on the β-carotene release data using SAS 9.3 (Cary, NC). Fortification type, digestion method, and digestion phase were all treated as fixed effects. Means were separated using a Tukey post hoc test ($p < 0.05$).

RESULTS AND DISCUSSION

Surface Morphology of Capsules. SEM images of a WPI-alginate-chitosan microcapsule at magnification ranging from 100× to 1000× are shown in Figure 1. At the 100× magnification (A), it was shown that the microcapsules were spherical. All of the images show that the chitosan surface of the microcapsules was rough, but did not exhibit cracking as a result of the drying process. The rough surface of the capsules did not appear to be caused by high porosity, but rather by surface irregularities. Larger surface irregularities were observed on some particles, which can be seen in images C and D. These protrusions may enhance the release properties of the capsules due to the increased surface area of the capsule. Overall, the capsules were observed to have no cracking and low porosity, which suggest high integrity of the encapsulating materials.[35]

***β*-Carotene Content of Almond Butter and Capsules.** The target and actual β-carotene content of almond butter samples and WPI-alginate-chitosan capsules tested are shown in Table 1. As expected, the control almond butter contained a small amount of β-carotene. The actual β-carotene content of oil fortified almond butter and encapsulated β-carotene oil was slightly less than the targeted concentration. This difference may be due to losses during preparation of samples or possible inconsistencies in the β-carotene content of the source, Lucarotin 30M.

pH of Almond Butter Digesta. All almond butter samples exhibited similar pH values. The pH of almond butter before in vitro digestion was 5.32 ± 0.02. Figure 2 shows the average pH profiles of almond butter samples in the two digestion models tested in this study, HGS and shaking water bath. The pH for these models was manually adjusted by the addition of 6 M HCl or 5 M NaOH after each phase of digestion (saliva, gastric pH 4, gastric pH 3, gastric pH 2, intestine). There were no significant differences in the pH profiles of the HGS and shaking water bath digestion models. After the addition of saliva (pH 7.0), the pH of the sample rose to approximately pH 6. After each pH adjustment made during the gastric phase of digestion, pH 4, 3, and 2, a slight increase in pH was observed. This rise in pH is attributed to the buffering effect of almond butter. For the intestinal phase of digestion that began at 120 min of digestion, the pH of the digesta was adjusted to 6.5. After 2 h of intestinal digestion, the pH of the digesta decreased to approximately 6.2. This decrease in the pH of digesta is attributed to the generation of free fatty acids by the action of pancreatic lipase, because a previous digestion method that measured release of free fatty acids by titration of intestinal digesta with NaOH and maintaining the pH at 7.0 made note of the pH changes of high lipid foods as a result of the generation of free fatty acids.[36] The decrease in pH during the intestinal phase of digestion may have also been attributed to the release of amino acids as a result of proteolysis.

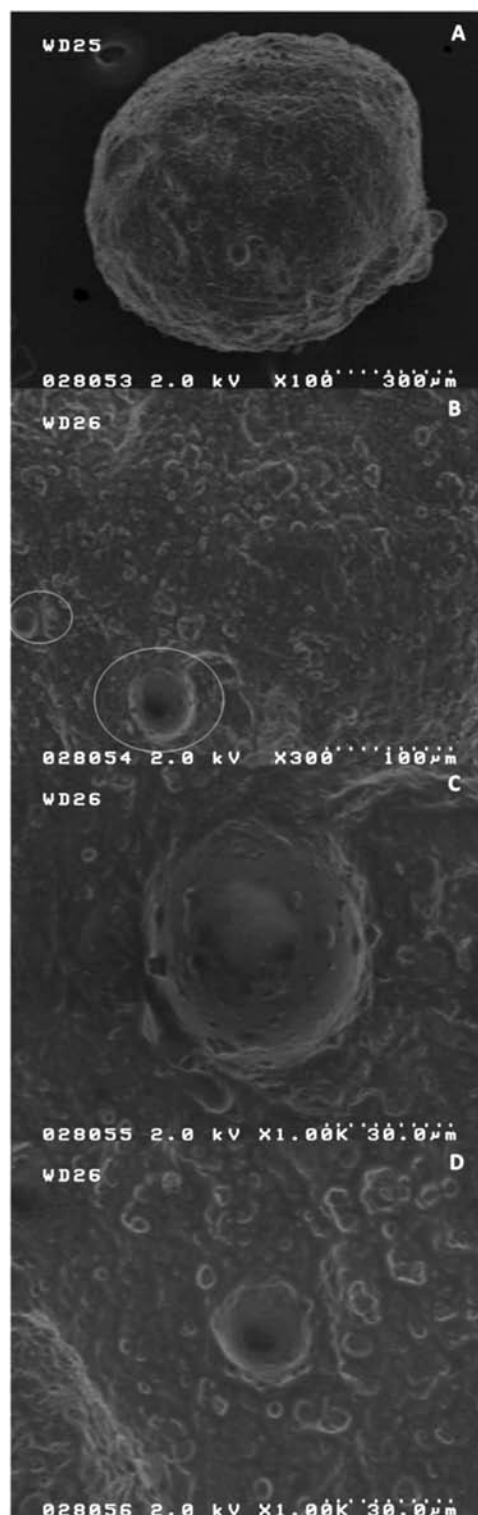

Figure 1. Scanning electron microscopy (SEM) photographs of alginate-chitosan microcapsule. (A) Capsule at 100× magnification, (B) capsule at 300× magnification, and (C,D) capsule at 1000× magnification. The areas that were magnified are circled.

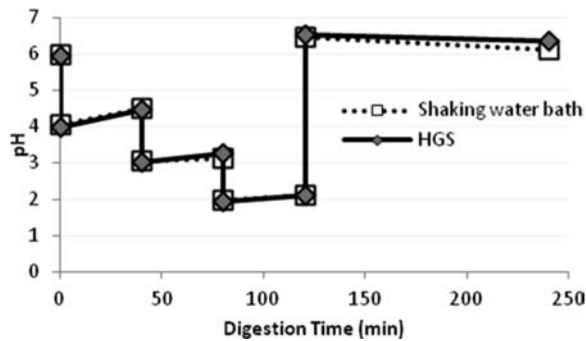

Figure 2. Comparison of pH changes during almond butter simulated digestions between digestion models.

Viscosity of Almond Butter Digesta. The viscosities of control and fortified almond butter were not significantly different and exhibited shear thinning behavior (data not shown). Therefore, the viscosity of almond butter control samples after each stage of digestion was measured to determine if there was a difference in the viscosity of samples obtained from the two digestion models, shaking water bath and HGS. Because the HGS model mimics peristalsis in the gastric phase of digestion, it was hypothesized that samples from this model might have a different viscosity due to greater mixing of digestive juices into the samples. All of the digestion samples exhibited shear thinning behavior (Figure 3). As expected, the viscosity of digestion samples decreased as digestion progressed due to the breakdown of macronutrients facilitated by acid and enzymatic reactions as well as the addition of digestion solutions. The higher viscosity of the HGS samples in the earlier stages of digestion (saliva and gastric pH 4) may possibly be attributed to the higher level of mixing of digestive juices for that method. Overall, the differences in the viscosities of digestion samples from the HGS and shaking water bath models were minimal.

***β*-Carotene Release during Digestion.** The release of *β*-carotene after different stages of digestion was quantified to determine if there were differences in release between the two fortification methods (oil and capsule) and two simulated digestion models (shaking water bath and HGS). For all of the spectrophotometer readings of *β*-carotene content, almond butter without fortification was used as a blank. A UV−vis spectrophotometric method of analyzing *β*-carotene content was chosen over reverse phase HPLC method because it is a more rapid technique with adequate detection thresholds. However, it should be noted that spectrophotometric readings do not detect *β*-carotene isomerization. Although isomerization during digestion is usually minimal,[37,38] it may impact bioavailability and may limit comparability with in vivo data.

Figure 4 shows the *β*-carotene release during simulated digestion (shaking water bath vs HGS) from oil fortified almond butter. *β*-Carotene was not completely released from the almond butter at any stage of digestion. This suggests that the protein or carbohydrate compounds in the almond butter food matrix may emulsify or encapsulate *β*-carotene that has been directly added to almond butter to control its release from the food matrix. For the gastric phases and the overall intestinal phase, the *β*-carotene release was higher in the HGS model than the shaking water bath model. The HGS model more efficiently mixed samples in gastric phase of digestion, which may have allowed for better diffusion of gastric enzymes into the almond butter to break down samples and improve the release of the *β*-carotene. However, there was less *β*-carotene present in the aqueous fraction of intestine samples in the HGS model and about the same in the micelle fraction. The similar amount of *β*-carotene present in the micelle fraction in both models may have been due to the limiting of micellarization by the almond butter. Almonds are high in dietary fiber,[39] which has been previous shown to inhibit micellarization of carotenoids.[40,41]

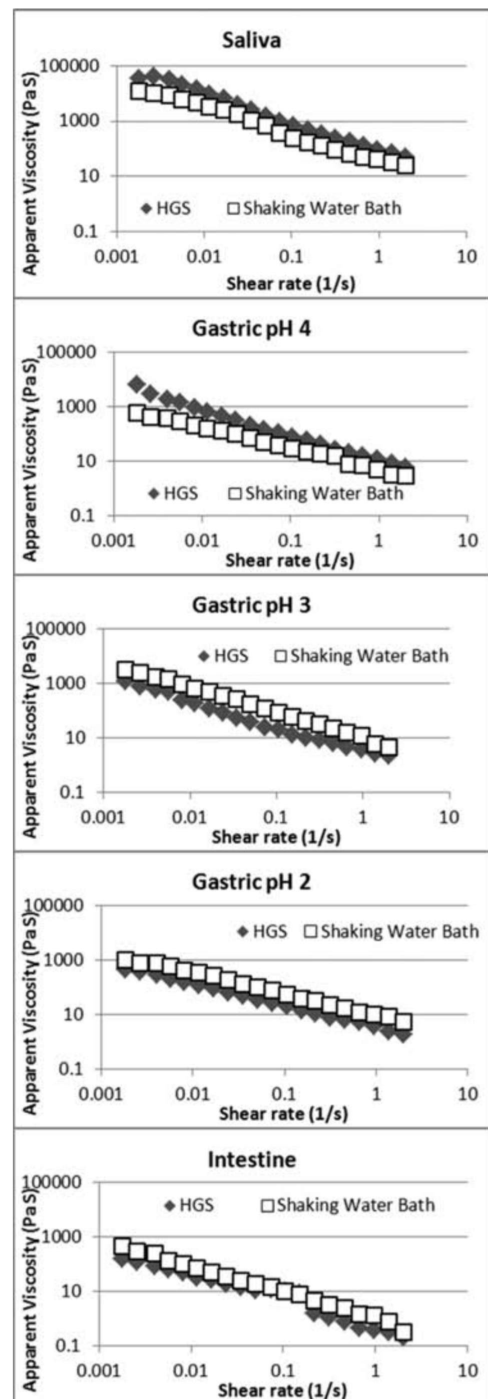

Figure 3. Shear rate (1/s) versus apparent viscosity (Pa·S) for digestion samples.

Figure 5 shows the *β*-carotene release during simulated digestion (shaking water bath vs HGS) from capsule fortified almond butter. Unlike the oil fortified almond butter, there was

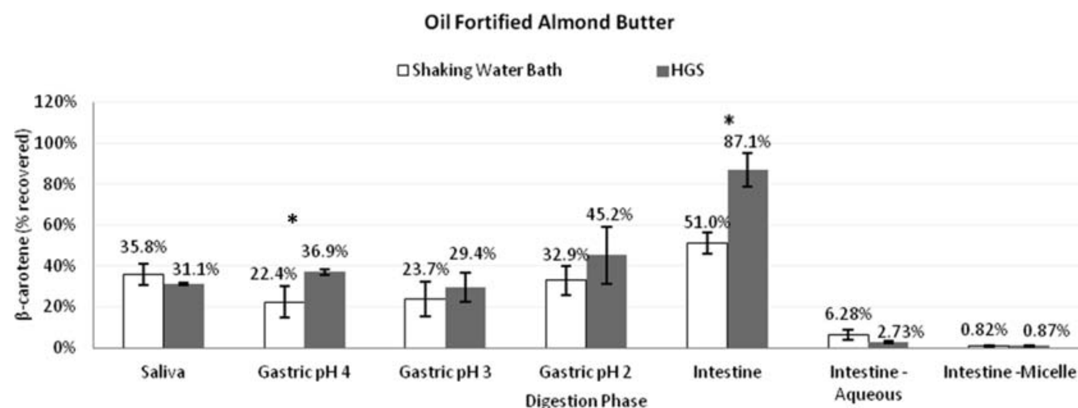

Figure 4. Shaking water bath and HGS digestion model comparison of percent β-carotene recovered at different stages of digestion from almond butter fortified with β-carotene oil (oil). Error bars denote standard deviation. Statistical difference ($p < 0.05$) between treatments is marked (*).

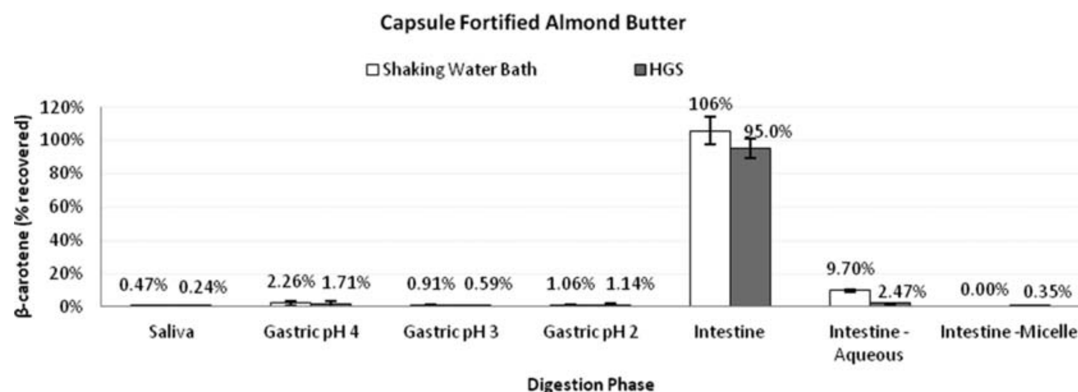

Figure 5. Shaking water bath and HGS digestion model comparison of percent β-carotene recovered at different stages of digestion from almond butter fortified with β-carotene capsule (encapsulated). Error bars denote standard deviation.

no significant difference in β-carotene release between the shaking water bath and HGS model for capsule fortified almond butter. For both the shaking water bath and the HGS digestion models, very little β-carotene was released (<2.26% released) until the intestinal phase of digestion, and thus peristalsis did not have a significant effect on the release of WPI-alginate-chitosan encapsulated β-carotene during gastric digestion. In addition, the capsules showed complete release (95.0 ± 5.8% and 106 ± 8.6%) into the intestinal digesta after 2 h of intestinal digestion. These data confirm that WPI-alginate-chitosan capsules' enteric release properties are not affected by gastric peristalsis in the presence of an almond butter food matrix. Further studies are recommended to determine the influence of gastric peristalsis on the release properties of microcapsules in the presence of a more solid food matrix. The additional force exerted in the presence of solid particles in stomach contents may cause early release of microcapsules targeted for intestinal delivery.

Although all of the β-carotene from capsule fortified almond butter was released in the intestinal phase, only a small portion was transferred to the aqueous and micelle fractions. More β-carotene was released in the aqueous fraction of the intestinal phase by the shaking bath model. However, more β-carotene was incorporated into the micellar fraction in the HGS model. In fact, for all of the shaking water bath samples, the β-carotene micelle fraction concentration was the same as the almond butter control.

The data collected from the in vitro digestion models indicate that WPI-alginate-chitosan encapsulation has a significant effect on the release of β-carotene from almond butter in the intestinal phase of digestion when compared to oil fortified almond butter ($p < 0.0001$). Although more β-carotene was recovered in the entire intestine phase for the WPI-alginate-chitosan samples (capsule, 106 ± 8.6% and 95.0 ± 5.8% recovered; oil, 51.0 ± 5.2% and 87.1 ± 8.2%, for shaking water bath and HGS, respectively), less β-carotene was incorporated into micelles for capsule fortification than oil fortification (capsule, 0 ± 0% and 0.35 ± 0.1% recovered; oil, 0.82 ± 0.3% and 0.87 ± 0.3%, for shaking water bath and HGS, respectively). Capsule fortified almond butter yielded less than one-half of the β-carotene in the micelles of oil fortified almond butter. However, the differences in micelle incorporation between the samples were not statistically significant.

The percent of β-carotene from all fortified almond butter that was incorporated into micelle fraction was much lower than that observed from natural sources of β-carotene, which have been previously measured to range from 1.30 ± 0.22% (pumpkin) to 17.45 ± 1.99% (boiled spinach).[42] This result was unexpected because previous studies have demonstrated a high percent of β-carotene transferred to micelles from high lipid foods. When examining bioaccessibility of β-carotene fortified canola oil, Wright et al.[29] observed greater than 50% β-carotene incorporated into micelles using the same intestinal phase that was used in this study. Malaki Nik et al.[43] demonstrated 48.4 ± 2.9% β-carotene transferred to micelles from a soy protein stabilized oil-in-water emulsion. Because measured transfer of β-carotene to micelles in almond butter

was low, fortification of almond butter with β-carotene is not recommended.

The observation that the release of β-carotene from capsules in the intestinal phase was significantly higher than oil fortified almond butter and that there was no significant difference in β-carotene micelle concentration between fortification methods suggests that WPI-alginate-chitosan may reduce the bioaccessibility of β-carotene. One possible explanation of the reduced β-carotene levels in the micelle fraction of capsule fortified almond butter is that alginate and/or chitosan may inhibit formation of micelles containing β-carotene. Yonekura et al.[40] observed reduced incorporation of β-carotene into micelles when digested in the presence of alginate. In addition, previous studies have indicated that alginate and chitosan can inhibit absorption of fat, which is a necessary component of mixed micelles. A human clinical trial that examined the effect of alginate on the ileostomy excretion of fat found that supplementation of diet with 7.5 g of sodium alginate increased fat excretion by 140%.[44] An in vivo trial using a rat model found that a diet containing 5% chitosan reduced fat digestibility by about 50% when compared to a control fiber, cellulose.[45] These findings merit further investigation on the impact of the physical and chemical properties of the material used for the shell of a microcapsule on the bioaccessibility of the bioactive that is encapsulated.

Differences in release of β-carotene between digestion models were only observed for the oil fortified almond butter. For this sample, high release was observed from the HGS model due to simulated peristalsis. For bioactives that do not need to be incorporated into mixed micelles to be absorbed, this difference in release may have a significant impact on assessment of bioaccessibility using a dynamic digestion model compared with a static digestion model. In the future, in vivo studies must be conducted in conjunction with static and dynamic in vitro models to determine which model more accurately simulates nutrient bioaccessibility.

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Notes

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ABBREVIATIONS USED

BHT, butylated hydroxytoulene; HGS, Human Gastric Simulator; HPLC, high performance liquid chromatography; RDA, recommended dietary allowance; RIVM, National Institute of Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, Bilhoven, The Netherlands); WPI, whey protein isolate

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