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In Vitro Gastric and Intestinal Digestion of a Walnut Oil Body Dispersion

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ABSTRACT: An oil body dispersion (11.3% fat) was prepared by wet disintegration of walnuts and was then subjected to a two-step model of *in vitro* digestion. In a gastric environment, proteolysis by pepsin led to the destabilization and coalescence of the oil bodies. Aggregation of the coalesced oil bodies was apparent under a confocal microscope, with aggregates up to 275 μm in size. Pepsin-resistant peptides and proteins remained at the surface of the oil bodies, and some were further resistant to intestinal proteases. Under intestinal conditions, the hydrolysis of walnut triglycerides led to the spontaneous formation of a new type of multiple emulsions, ranging from 2 to 45 μm in size and with protein material inside the inner water droplets. Transmission electron microscopy revealed the presence of a liquid-crystalline phase of bile salts and lipolytic products at the surface of the oil droplets and some bile salt crystals at the surface of the inner water droplets.

KEYWORDS: *Walnut oil bodies, in vitro digestion, confocal microscopy, transmission electron microscopy, multiple emulsion*

INTRODUCTION

Walnuts rank fifth in the production of all tree nuts, with China being the main producer.[1] They are very rich in lipids, about 64.5 g/100 g,[2] and contain high levels of phytosterols (mainly as β-sitosterol, campesterol, and stigmasterol) and polyunsaturated fatty acids (PUFA). Walnut lipids also contain tocopherols (mostly as γ-tocopherol), phospholipids (0.4% phosphatidylserine, 0.3% phosphatidylinositol, and 0.4% phosphatidylcholine), and sphingolipids (0.6%).[3] The main walnut fatty acids are myristic, ω-9 oleic, ω-6 linoleic, and ω-3 α-linolenic acids.[3] About 73% of the walnut fatty acids are polyunsaturated, and about 15% of the walnut fatty acids are monounsaturated.[2]

Walnuts contain about 13.5% proteins,[2] which are albumin (6.81%), globulin (17.57%), prolamin (5.33%), and glutelin (70.11%).[4] These proteins show minimal solubility at pH 4 and an increased solubility at pH <3 and >6. These four main proteins are composed of several polypeptides, with a molecular weight ranging from 12 to 67 kDa.[4] Some walnut proteins are allergenic.[5−7] One main property of food allergens is their stability for gastric digestion and, therefore, their ability to reach the intestine, where absorption and sensitization occur in some subjects.[8]

The *in vitro* and *in vivo* digestion of lipids and their associated health effects is the subject of extensive research.[9−12] The consumption of walnuts, because of their high level of PUFA and phytosterols, has been shown to decrease plasma total and low-density lipoprotein cholesterol.[13] Walnut intake has also been associated with beneficial effects on risk factors of cardiovascular disease, cancer, metabolic syndrome, and type 2 diabetes.[1] Walnuts, as a rich source of α-linolenic acid, reduced inflammation markers in hypercholesterolemic subjects.[14] Unfortunately, most studies do not consider the structure of walnut lipids in their native environment and during digestion as a critical parameter for their digestion and absorption.

Walnut lipids have been extensively characterized,[3,4,14] but their structure and behavior during digestion are poorly studied. In plants, seeds, and nuts, lipids are stored in oleosomes or oil bodies, which are oil droplets surrounded by a monolayer of phospholipids and embedded proteins called oleosins.[15] The microstructure of lipids will affect the way in which they are digested and absorbed by the body.[16] The stability[17,18] and digestion[19−22] of oil bodies have recently gathered some attention, because oil bodies are natural delivery vehicles of fat-soluble nutrients. Similar to our previous studies on almond oil body dispersions and bovine milk fat globules,[22,23] an aqueous dispersion of walnuts was obtained and then subjected to simulated gastrointestinal digestion. The aqueous dispersion of walnuts contained both walnut lipids and proteins (mostly storage proteins) in a matrix close to the bolus obtained after chewing walnuts prior to swallowing. This study aims at understanding the structural changes occurring during the digestion of walnut oil bodies.

MATERIALS AND METHODS

Chemicals. Shelled and dried walnut halves (nutritional composition as stated on the packaging: fat, 65.2%; protein, 15.2%; carbohydrate, 13.7%; origin, USA; fresh walnut flavor; and free from off-flavors and odor) were purchased fresh from a local wholesale retailer in Palmerston North, New Zealand. Pepsin from porcine gastric mucosa (EC 3.4.23.1, P7000, 800−2500 units/mg of protein), dried porcine bile extract (B8631), and porcine pancreatin (P1750, 4× United States Pharmacopeial specifications) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich Corporation, unless specified otherwise.

Preparation of Walnut Dispersions. Similar to the first processing steps of soy milk, a 250 g sample of walnuts was soaked overnight in 1 L of Milli-Q water (18.2 MΩ cm, purified by treatment with a Milli-Q apparatus, Millipore Corporation, Bedford, MA) at room temperature. The walnuts in water were then mixed in a wet

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disintegrator (Jeffress Bros, Ltd., Brisbane, Australia) for 5 min. The aqueous dispersion of walnut oil bodies and proteins, called in this work "walnut milk", was obtained after sieving the crushed mixture through a 150 μm powder sifter. Sodium azide (0.02%, w/v; Merck, Darmstadt, Germany) was added, and the milk was kept at 4 °C for a maximum of 2 days. The resulting walnut milk had a pH of 6.1 ± 0.1 and contained 11.3% fat, 3.1% protein, and 14.6% total solids content.

***In Vitro* Gastric Digestion.** The *in vitro* gastric digestion protocol simulated fasted-state conditions in humans with a pH between 1 and 3.[24,25] First, 10 mL of simulated gastric fluid (SGF) (per liter, 2 g of NaCl and 7 mL of HCl at pH 1.2[26]) was mixed with 20 mL of milk, which was then acidified to pH 1.5 with 6 M HCl and incubated at 37 °C for 10 min in a shaking water bath at 95 revolutions/min. Then, pepsin was added at the physiologically relevant substrate/enzyme ratio (w/w) of 20:1, and the temperature and pH were kept constant for 1 h. No gastric lipase was used for reasons explained elsewhere.[22] Samples were collected periodically for further characterization.

***In Vitro* Intestinal Digestion and Free Fatty Acid Release.** The simulated intestinal fluid (SIF) (per liter, 6.8 g of K_2HPO_4 and 190 mL of 0.2 M NaOH at pH 7.5[27]) contained 150 mM NaCl to simulate the *in vivo* intestinal ionic strength and bile extract (5 mg/mL). No calcium was added because bile extract may already contain physiological concentrations of calcium.[28] Walnut milk was subjected to gastric digestion for 1 h, as described above. Then, the *in vitro* intestinal digestion was carried out by mixing the digested walnut milk with SIF (1:3, v/v) to a total of 30 mL in a conical flask. The pH was adjusted to 7, and the mixture was placed in a shaking water bath (95 revolutions/min) at 37 °C. Pancreatin (1.6 mg/mL) was added to the mixture; the pH was maintained at 7 with 1 M NaOH; and samples were taken periodically over 2 h for characterization.

The activity of pancreatic lipase was measured over 2 h using a pH-stat titration method (TitraLab 856, Radiometer Analytical, Villeurbanne, France) with 0.05 M NaOH and an end point of pH 7.0. The total free fatty acids released were back-titrated at different time points of intestinal digestion, as described elsewhere.[22,29] A blank titration without pancreatin was carried out.

ζ Potential and Particle Size Distribution. The ζ potential and average droplet size of the walnut milk and digested samples were measured using Malvern Zetasizer Nano ZS and Mastersizer MSE instruments (Malvern Instruments, Ltd., Worcestershire, U.K.), respectively, as described elsewhere.[29] Walnut oil has a refractive index of 1.461[4] with an absorbance of 0.001, and the refractive index of the aqueous phase is 1.33. Triplicate measurements were made on at least three individual digestion experiments.

Gel Electrophoresis. The protein composition of digested walnut milk samples under gastric and intestinal conditions was determined by reducing tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), as described elsewhere;[29] 15 μL of gastric samples, 30 μL of intestinal samples, and 10 μL of standards were loaded on the gel.

Confocal Laser Scanning Microscopy (CLSM). A confocal laser scanning microscope (Leica DM6000B, Heidelberg, Germany) was used to study the microstructure of walnut milk oil bodies and gastric and intestinal samples, as described elsewhere,[30] using Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one, 1 mg/mL in dimethyl sulfoxide, 1:100, v/v) to stain the triglycerides and Fast Green FCF (disodium 2-[[4-[ethyl-[(3-sulfonatophenyl)methyl]amino]phenyl]-[4-[ethyl-[(3-sulfonatophenyl)methyl]azaniumylidene]cyclohexa-2,5-dien-1-ylidene]methyl]-5-hydroxybenzenesulfonate, 1 mg/mL in Milli-Q water, 1:100, v/v) to stain the proteins.

Transmission Electron Microscopy (TEM). A Philips 201C transmission electron microscope (Philips, Amsterdam, The Netherlands) was used to investigate the structure of the samples after digestion under gastric and intestinal (60 or 120 min) conditions. Digested milk samples were injected into freshly made 3% agarose tubes. The tubes were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The tubes were then fixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in acetone, and embedded in fresh 100% resin. The resin blocks were cut using a diamond knife and an Ultramicrotome (Leica, Vienna, Austria) and were stained with Toluidine Blue for viewing under a light microscope (Olympus BX51, Tokyo, Japan). Ultrathin sections were collected on a copper grid and were stained with saturated uranyl acetate in 50% ethanol and then with lead citrate.

RESULTS AND DISCUSSION

Particle Size Distribution and ζ Potential. The particle size of the oil bodies was affected during digestion (Figure 1).

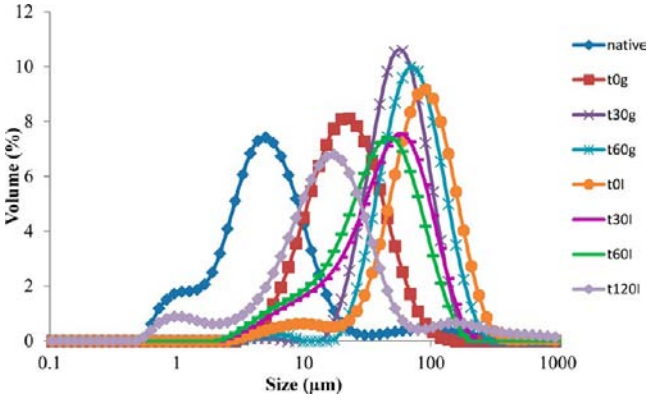

Figure 1. Particle size distributions of walnut oil bodies before (native) and during gastric (t0g, before adding pepsin; t30g, 30 min after adding pepsin; and t60g, 60 min after adding pepsin) and intestinal (t0I, before adding pancreatin; t30I, 30 min after adding pancreatin; t60I, 60 min after adding pancreatin; and t120I, 120 min after adding pancreatin) digestion.

The walnut oil bodies had a Sauter average diameter, d_{32}, of 5.8 ± 0.1 μm and a volume mean diameter, d_{43}, of 9.8 ± 0.5 μm. Walnut oil bodies showed a bimodal size distribution, with particle sizes between 0.5–1.4 and 1.4–30 μm (Figure 1). The larger particles may result from the coalescence of smaller oil bodies during the walnut milk preparation. A similar bimodal distribution was observed when the walnut milk was mixed with SGF. The smaller oil bodies are likely to be digested faster than the larger oil bodies[9] and, consequently, were not detected after 30 min of gastric digestion (Figure 1). During gastric digestion, the size distribution shifted toward larger sizes (10–275 μm), whereas, under intestinal conditions, the distribution of the particle size became trimodal (1.6–45 and 45–360 μm) with the increasing detection of particles in the size range of 0.4–1.6 μm (Figure 1), possibly because of the formation of micelles and vesicles containing lipolytic products. In *in vivo* models, vesicles and micelles are usually found to be relatively small (in the order of a few nanometers up to 600 μm[11]), but in *in vitro* conditions, they may coalesce because of their accumulation in the reaction chamber.[22]

The walnut oil bodies carried a negative charge at neutral pH (Figure 2). When subjected to gastric conditions, the surface charge became positive because of the decrease in pH (Figure 2). The ζ potential dropped quickly (i.e., became less positive) after the addition of pepsin because of the hydrolysis of proteins present at the oil body surface by pepsin. As a result, the repulsion between oil bodies decreased, which led to their aggregation. After the addition of bile extract and an increase in pH (intestinal conditions), the ζ potential of the oil bodies decreased further (Figure 2), indicating the adsorption of negatively charged bile salts to the interface. A further gradual drop in ζ potential (Figure 2) was probably caused by the accumulation of lipolytic products at the oil–water interface.

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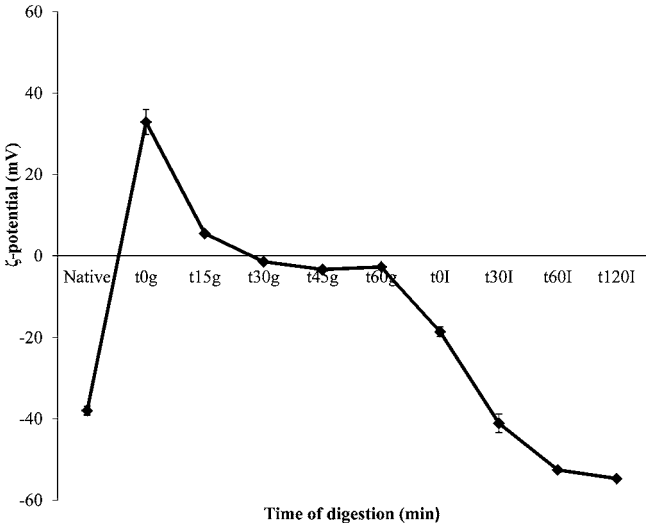

Figure 2. ζ-Potential values of walnut oil bodies before (native) and during gastric (t0g, before adding pepsin; t15g, 15 min after adding pepsin; t30g, 30 min after adding pepsin; t45g, 45 min after adding pepsin; and t60g, 60 min after adding pepsin) and intestinal (t0I, before adding pancreatin; t30I, 30 min after adding pancreatin; t60I, 60 min after adding pancreatin; and t120I, 120 min after adding pancreatin) digestion.

***In Vitro* Protein Hydrolysis.** The hydrolysis of walnut proteins by pepsin and the intestinal proteases was followed by reducing tricine–SDS–PAGE (Figure 3). The native walnut storage polypeptides were identified following Sze-Tao and Sathe's nomenclature.[4] Walnut oleosins, which present a major band of 12 kDa and three minor bands of 3, 20, and 62 kDa,[31] have similar molecular weights to those of walnut storage proteins and, hence, cannot be differentiated on the SDS–PAGE (Figure 3). Most proteins were still present after 15 min of digestion by pepsin (Figure 3). At the end of the 60 min of gastric digestion, the main walnut protein, glutelin (G) (strong doublet at 33 and 37 kDa and another major polypeptide at 21 kDa), some pepsin-resistant peptides, and possibly some minor low-molecular-weight (<10 kDa) polypeptides of albumin (A), prolamin (P), and globulin (B) were still detected (Figure 3). The pepsin-resistant peptides were easily hydrolyzed by intestinal proteases. However, a faint band of glutelin (33 kDa) was still detected at the end of the intestinal digestion (Figure 3).

After extraction and purification, walnut glutelin was found to be easily hydrolyzed *in vitro* by pepsin, trypsin, and chymotrypsin, whether in its native form or its heat-denaturated form.[4] The method to isolate glutelin possibly changed its conformation and exposed sites prone to hydrolysis by gastrointestinal proteases. Another explanation of the dissimilarity between our results and the results found for isolated glutelin[4] may be the effect of the interactions between walnut phenolic compounds and proteins at neutral and alkaline pH. The skin of walnuts is very rich in phenolic compounds, mainly condensed tannins and phenolic acids.[32] The interactions of walnut proteins with phenolic compounds have been shown to decrease their solubility, having a possible effect on the protein bioavailability.[32] Therefore, these phenolic–protein interactions, mainly ionic and hydrophobic, may have been responsible for the partial resistance of walnut glutelin to proteolysis, because walnuts with skin were used for the preparation of walnut milk. One last explanation may be the presence of polysaccharides and insoluble fibers from walnut skins interacting with walnut proteins by covalent bonding or electrostatic interactions[33] and having a similar protective effect on the proteins against hydrolysis.

Free Fatty Acid Release. The release of total free fatty acids was typical of that of an oil-in-water emulsion (Figure 4).

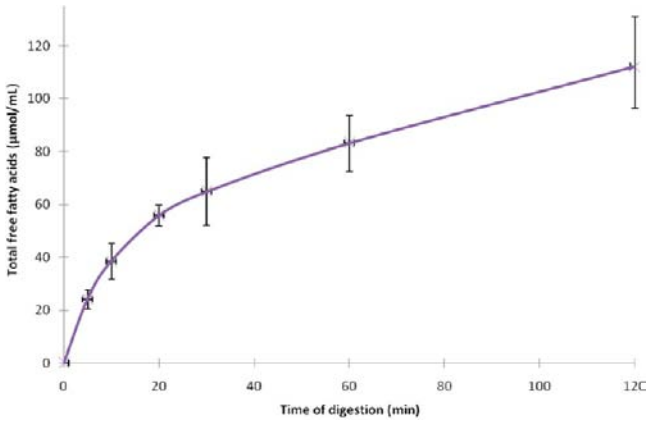

Figure 4. Total free fatty acid release from gastric-digested walnut milk oil bodies during 120 min of intestinal digestion and measured after back-titration.

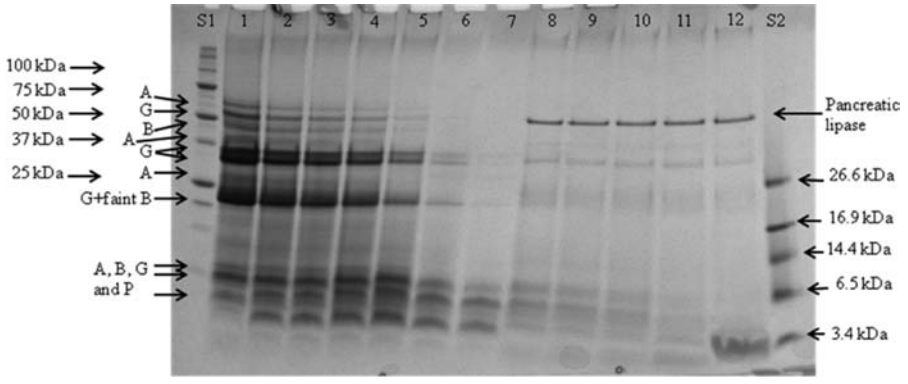

Figure 3. Reducing tricine–SDS–PAGE patterns of walnut milk and digested samples. Identification of strong and faint bands of proteins according to Sze-Tao and Sathe.[4] A, albumin; B, globulin; G, glutelin; P, prolamin; S1, high-molecular-weight standard; 1, t0g; 2, t1g; 3, t2g; 4, t5g; 5, t15g; 6, t30g; 7, t60g; 8, t1I; 9, t2I; 10, t5I; 11, t15I; 12, t120I; S2, low-molecular-weight standard; g, gastric; and I, intestinal.

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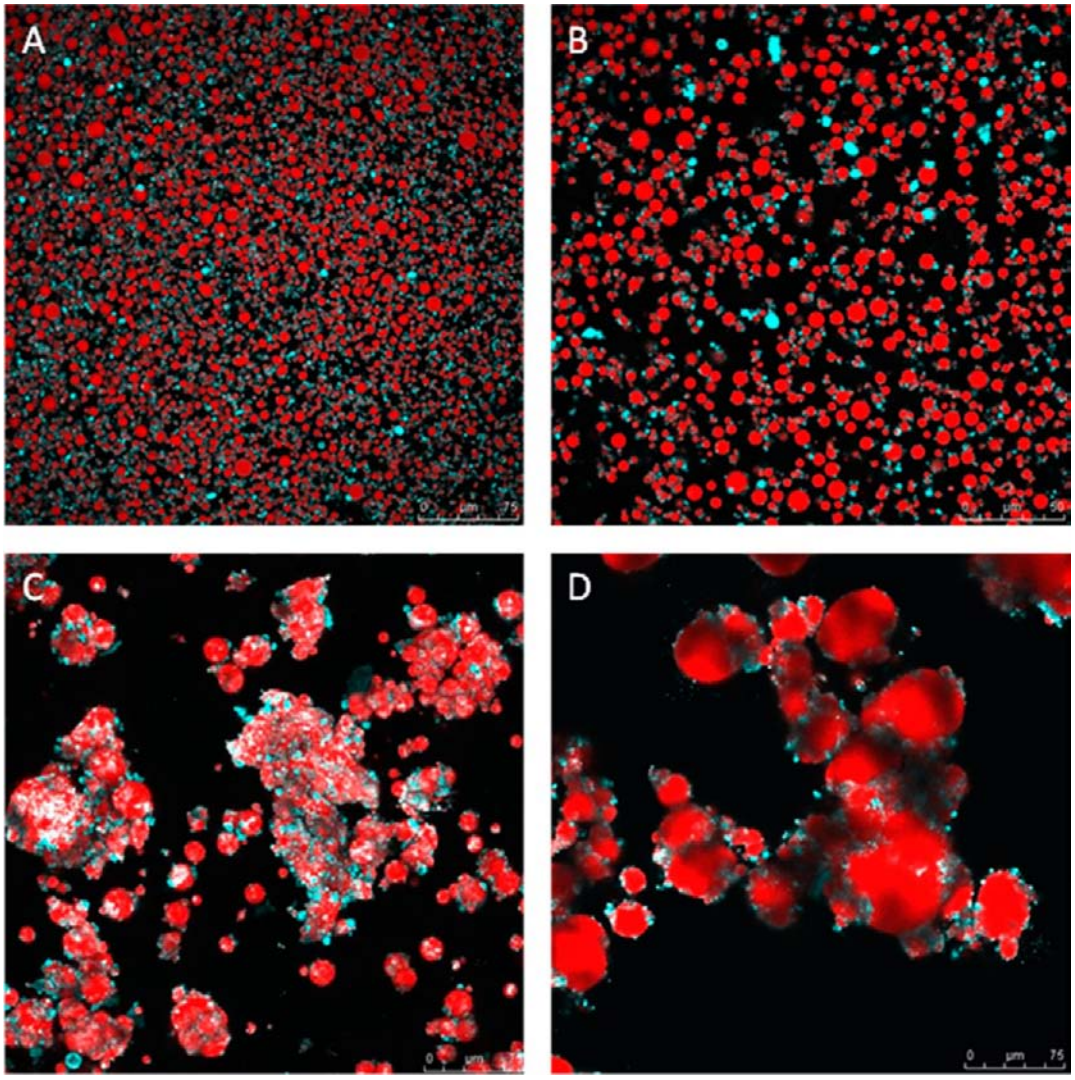

Figure 5. CLSM images of native (A) and digested (B, 0 min; C, 30 min; and D, 60 min) walnut oil bodies under simulated gastric conditions with (C and D) or without (A and B) pepsin. The triglyceride core of the oil bodies was stained with Nile Red (red), and the proteins were stained with Fast Green FCF (blue). Scale bars = 50 μm (B) and 75 μm (A, C, and D).

The rate of lipolysis was fast in the first 10 min and then slowed because the accumulation of lipolytic products at the interface prevented the pancreatic lipase from accessing the triglyceride core.[29] These lipolytic products are further solubilized into phospholipid vesicles and mixed phospholipid−bile salt micelles.[34] No lag phase was observed in the titration curve of the release of titrable free fatty acids (results not shown), suggesting that pancreatic lipase was rapidly adsorbed and activated at the surface of the walnut oil bodies. The rate of lipolysis slowed down progressively after 10 min and could be accelerated in the presence of higher concentrations of calcium,[35] because calcium associates with long-chain fatty acids to form insoluble soaps, which precipitate from the oil−water interface, enhancing access of pancreatic lipase to the triglyceride core.[36]

Microstructural Changes. *CLSM.* The microstructure of the native walnut milk under CLSM was typical of that of an oil-in-water emulsion, with oil droplets surrounded by a layer of proteins (Figure 5). Few oil bodies aggregated when the walnut milk was mixed with the acidic SGF. Once proteolysis by pepsin started, large oil bodies were observed (Figure 5), indicating their coalescence because of destabilization of the interface and incomplete coverage by peptides from proteolysis. The coalesced oil bodies formed aggregates, as expected from the low ζ potential of the oil bodies during gastric digestion. The aggregation of large oil bodies also explains the increase in particle size during *in vitro* digestion (Figure 1). Their surface was covered by pepsin-resistant peptides and proteins (Figure 5).

Once the pepsin-digested walnut milk was mixed with SIF and bile salts at pH 7, breakdown of the oil body aggregates and stabilization of the walnut oil bodies (Figure 6) occurred because of the adsorption of negatively charged bile salts at their surface, providing electrostatic repulsions between droplets. An unexpected phenomenon occurred after the addition of pancreatin. The formation of a new type of water-in-oil-in-water emulsion, trapping protein material stained with Fast Green FCF (panels B−D of Figure 6) in the inner water droplets, was observed with an increasing yield with the time of digestion (Figure 6). This new structure will be called a "spontaneous biological multiple-phase emulsion" (SBMPE).

The formation of a SBMPE was not observed in our previous studies of the *in vitro* digestion of natural oil-in-water

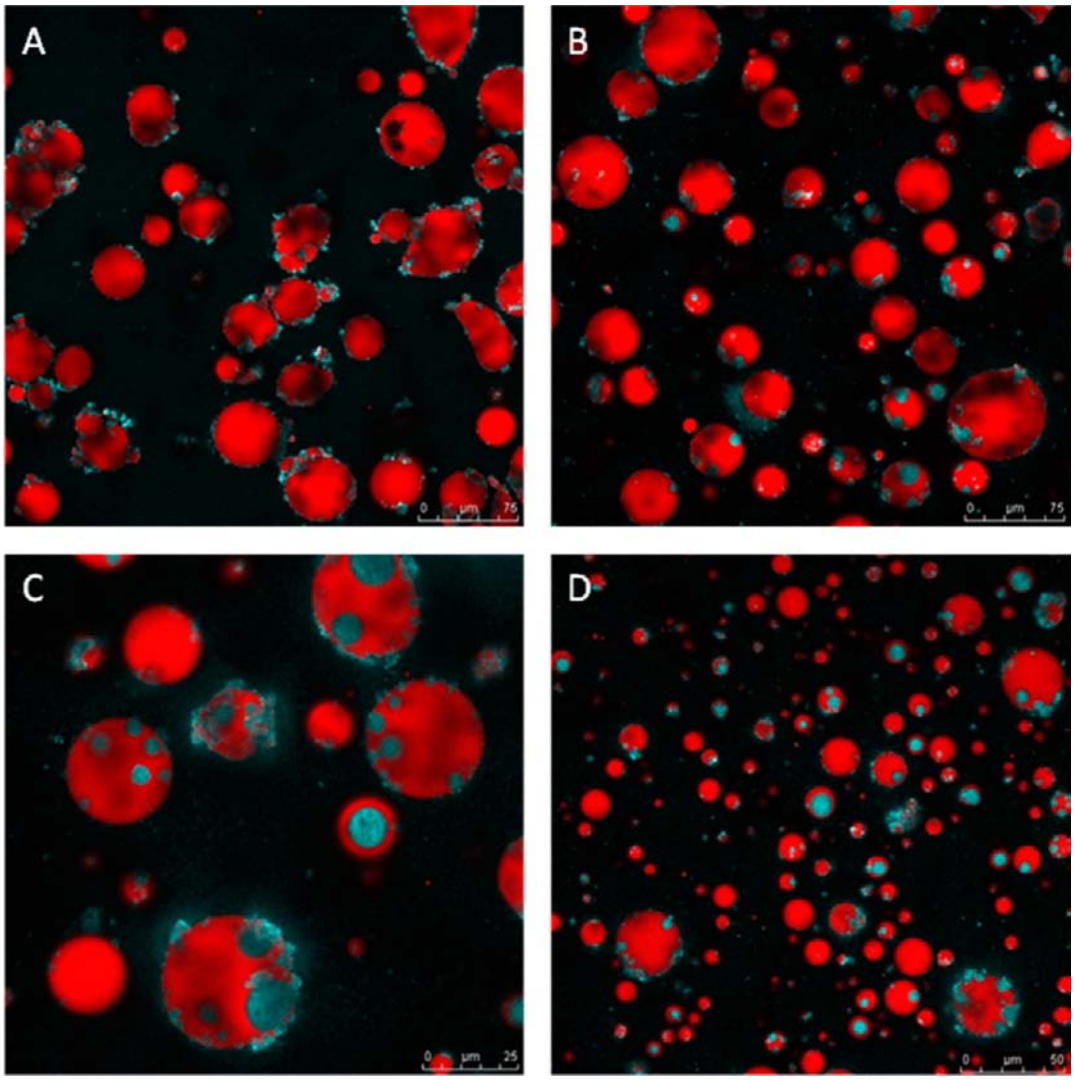

Figure 6. CLSM images of digested walnut oil bodies under simulated intestinal conditions after 1 h of gastric digestion with (B, 30 min; C, 60 min; and D, 120 min) or without (A) pancreatin. The triglycerides were stained with Nile Red (red), and the proteins were stained with Fast Green FCF (blue). Scale bars = 25 μm (C), 50 μm (D), and 75 μm (A and B).

emulsions, the almond oil bodies, and bovine milk fat globules, using similar conditions.[29,37] It was not observed when the pepsin-digested walnut milk was mixed with SIF in the presence of only either bile extract or pancreatin (results not shown). Therefore, the particular fatty acid profile of walnuts, rich in PUFA, interacting with bile salts, may have been responsible for the "spontaneous" formation of a multiple-phase emulsion upon the release of free fatty acids. Added fluorescent dyes and agarose were not responsible for the formation of the SBPME because it occurred in a similar way without any dyes or agarose (results not shown). It is surprising to observe this phenomenon because the agitation was rather gentle, whereas the typical processing of double emulsions requires high shear emulsification. Double emulsions have been observed during phase inversion in the presence of a balanced combination of hydrophilic and lipophilic emulsifiers.[38] This could have been the driving factor for the formation of the SBMPE. Indeed, under *in vitro* conditions of the intestinal digestion of walnuts, most free fatty acids (mostly long-chain fatty acids in walnuts) are partially deprotonated,[39] the bile contains negatively charged bile salts, amphiphilic phospholipids, lipophilic cholesterol, and electrolytes,[40] and some walnut peptides are still present. As explained previously,[30] soaking nuts leads to the activation of a phospholipase D that is responsible for the transphosphatidylation of phosphatidylethanolamine and phosphatidylcholine into phosphatidic acid. Phosphatidic acid carries a negative charge and is very hydrophilic at neutral pH[41] and, therefore, has a preference for the water phase.

TEM. The digested walnut samples after 60 min (Figure 7) and 120 min (Figure 8) under intestinal conditions were analyzed using TEM. The double emulsion shape was kept during sample preparation (panels A–C of Figure 7 and panel C of Figure 8). The lipolytic products were transported in either vesicles or mixed micelles (black arrows in panels A, C, and D of Figure 7 and panels B and C of Figure 8). Both the SBMPE and multiple-phase micelles contained electron-dense material inside the inner water droplets (panels A–C of Figure 7 and panel C of Figure 8), as seen in panels B–D of Figure 6. This electron-dense material or water–protein-rich phase could have been either walnut pepsin-resistant peptides or gastric and intestinal enzymes.

However, the SBMPE was different from the typical double emulsions, which usually require two types of emulsifier, one to

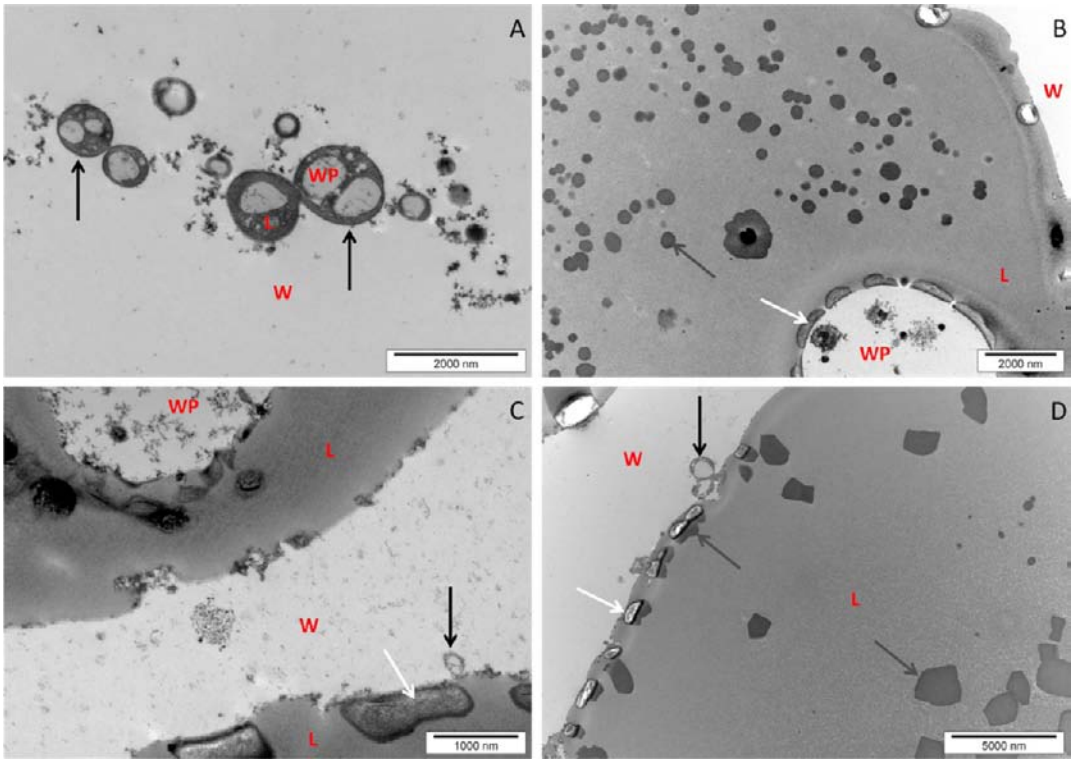

Figure 7. TEM images of digested samples after 60 min of gastric digestion followed by 60 min of intestinal digestion. Black arrows are pointing at vesicles and micelles transporting lipolytic products. White arrows are pointing at bile salt crystals. Gray arrows are pointing at lipolytic product crystals. W, water phase; L, oil phase; and WP, water−protein-rich phase.

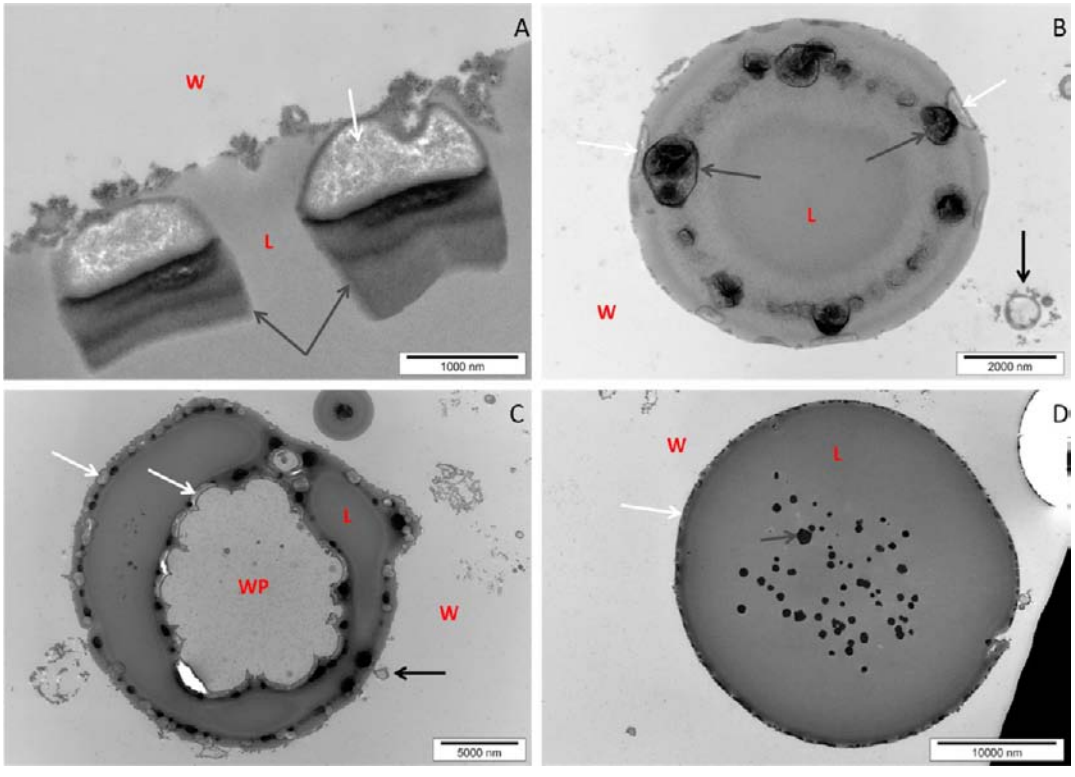

Figure 8. TEM images of digested samples after 60 min of gastric digestion followed by 120 min of intestinal digestion. Black arrows are pointing at vesicles and micelles transporting lipolytic products. White arrows are pointing at bile salt crystals. Gray arrows are pointing at lipolytic product crystals. W, water phase; L, oil phase; and WP, water−protein-rich phase.

stabilize the inner droplet and one to stabilize the outer droplet.[38] Indeed, as seen in Figures 7B and 8C, similar crystals were found at both interfaces. These crystals could have been composed of bile salts, phospholipids (from bile extract or from

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walnut oil bodies),[42] calcium-fatty acid soaps, monoglyceride soaps,[43,44] or a combination of some of them. The most likely candidates are the biosurfactants, bile salts, which, with their hydrophobic side and hydrophilic side, tend to lay flat at the surface of oil droplets.[45] They tend to associate into disk-shaped micelles;[46] their convex side interacts with the concave side of the colipase at the oil−water interface. Bile salts and lecithin have been shown to form liquid crystals at the oil−water interface, and bile salts form a surfactant phase with monoglycerides in the presence of NaCl when the hydrophile−lipophile property of the two mixtures is just balanced.[42] In the presence of surface-active molecules, a liquid-crystalline phase can occur when the molecule concentration increases from a dilute system to a saturated solution.[47] Thus, it can be assumed that the increased concentrations of adsorbed bile salts at the oil−water interface and lipolytic products, i.e., free fatty acids and monoglycerides, can lead to the formation of a liquid-crystalline phase at the oil−water interface as lipolysis takes place and before bile salts form micelles, solubilizing the lipolytic products.[48,49] Anionic detergents transition from a liquid-crystalline phase to a micellar phase upon an excess of molecules.[45] This could explain why no crystals were observed in the micelles (Figures 7A and 8B).

In addition, no calcium was added to the SIF but is likely present in the bile extract. Under *in vivo* conditions, long-chain fatty acids are precipitated by calcium into insoluble soaps; therefore, in the presence of small concentrations of calcium, long-chain fatty acids tend to accumulate at the interface, forming a hydrated liquid-crystalline phase.[12,47] The crystals present in the oil phase may be the lipophilic emulsifiers migrating in the oil phase,[38] in the present case, the lipolytic products. These crystals (gray arrows in panels B and D of Figure 7 and panel D of Figure 8) tended to migrate toward the oil−water interface and to associate with bile salt crystals (white arrows), as seen in panel D of Figure 7 and panels A and B of Figure 8. One can hypothesize that some of these interfacial crystals contain some calcium.

Dependent upon the ratio of lipids/lipolytic products/bile salts/cholesterol/phospholipids present during digestion, several structure formations, such as lamellar liquid crystals, mixed micelles, and mutilamellar or unilamellar vesicles, can be observed.[40] Interfacial liquid crystals were observed in the presence of surfactants and electrolytes, such as sodium chloride.[50,51] In some instances, a decrease in the interfacial tension could result in the formation of liquid crystals at the water−oil interface by orienting hydrophilic surfactants.[51] It is thought that bile salts interact synergistically with phospholipids at the surface of oil droplets in the duodenum, causing a reduction in the interfacial tension.[52] Water-in-oil-in-water emulsions could be formed in the liquid-crystalline-like detergent phase of surfactants, where there is a contrasting balance between the lipophilicity and the hydrophilicity of the surfactants.[51] This could have been the main factor here for the formation of crystals of amphiphilic bile salts at the water−oil and oil−water interfaces.

The present study showed the *in vitro* digestion of walnut lipids and proteins using a static model that simulated gastric and intestinal conditions. The digestion of walnut proteins was not complete, and the walnut oil bodies coalesced and formed aggregates under gastric conditions. In the intestinal environment, a SBMPE was formed, with bile salt crystals stabilizing the water−oil and oil−water interfaces. These structures are likely to play a major role in how lipids are digested and absorbed from walnuts. In the future, we plan to look at the digestion in a dynamic model that considers the buffering capacity of the food, the gradual secretion of gastric and intestinal juices, the gastric emptying rate, and the intestinal absorption of products of digestion. Another important parameter to consider is the effect of adding a gastric lipase during the gastric phase. It will be of great value to compare the results of *in vitro* digestion to *in vivo* data to determine if the formation of a SBMPE occurs *in vivo*.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CLSM, confocal laser scanning microscopy; PUFA, polyunsaturated fatty acids; SBMPE, spontaneous biological multiple-phase emulsion; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TEM, transmission electron microscopy

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