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Release of Protein, Lipid, and Vitamin E from Almond Seeds during Digestion

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The evaluation of the bioaccessibility of almond nutrients is incomplete. However, it may have implications for the prevention and management of obesity and cardiovascular disease. This study quantified the release of lipid, protein, and vitamin E from almonds during digestion and determined the role played by cell walls in the bioaccessibility of intracellular nutrients. Natural almonds (NA), blanched almonds (BA), finely ground almonds (FG), and defatted finely ground almonds (DG) were digested in vitro under simulated gastric and gastric followed by duodenal conditions. FG were the most digestible with 39, 45, and 44% of lipid, vitamin E, and protein released after duodenal digestion, respectively. Consistent with longer residence time in the gut, preliminary in vivo studies showed higher percentages of nutrient release, and microscopic examination of digested almond tissue demonstrated cell wall swelling. Bioaccessibility is improved by increased residence time in the gut and is regulated by almond cell walls.

KEYWORDS: Almonds; bioaccessibility; digestion models; cell walls

INTRODUCTION

Recent studies have demonstrated positive effects of nut consumption in modifying lipid risk factors for coronary heart disease (CHD) (*1*−*6*). Consumption of nuts, including almonds, is recommended by the current American Heart Association guidelines to replace saturated fats with unsaturated fats (*7*) and is compatible with the National Cholesterol Education Program (NCEP) guidelines for increasing the proportion of monounsaturated fat in the total amount of fat consumed (*8*). In a recent dose−response study, almonds significantly reduced CHD risk factors, and this was attributed to the high levels of protein, dietary fiber, and lipids rich in oleic acid (*9*). Incorporation of almonds into the diets of healthy and mildly hypercholesterolemic adults markedly improved the serum lipid profile (*10*). Compared to other tree nuts, almonds are unusual for the large amounts of protein and α-tocopherol they contain, and incorporating almonds into the diet increased α-tocopherol concentrations in red blood cells and lipid-adjusted plasma (*11, 12*). Vitamin E, defined as α-tocopherol by the Food and Nutrition Board of the Institute of Medicine, is known as a potent lipophilic antioxidant and stabilizer of membranes (*13, 14*). A recent study suggested that an antioxidant therapy combining vitamin C with vitamin E gave beneficial effects after myocardial infarction (*15*). In 2000, the Recommended Dietary Allowance for vitamin E in the United States increased from 10 to 15 mg of α-tocopherol per day for adult men and women (*16*).

The main component of almond seeds is lipid (50−55%). However, it has previously been reported that a significant proportion of lipid material remains undigested in healthy human volunteers incorporating peanuts or almonds into their diet (*17, 18*). We have previously demonstrated that almond cell walls (dietary fiber) play an important role in reducing lipid bioaccessibility (*19*). The term "bioaccessibility" is defined as the proportion of a nutrient that can be released from a complex food matrix and therefore becomes potentially available for absorption in the gastrointestinal (GI) tract (*19*). Studies of bioaccessibility are of importance in the areas of disease prevention and management (e.g., obesity and CHD). For example, the rate of

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lipid digestion and absorption is considered to be an important factor in influencing postprandial lipemia, which is now thought to influence the atherosclerotic process involved in CHD (*20, 21*). A delay in the postprandial rise in plasma triacylglycerol concentration in volunteers fed whole almonds has been demonstrated, and the possible link between lipid bioaccessibility and fat-stimulated satiety is strongly related to cholecystokinin (CCK) release (*22*).

Although we have recently examined the role of almond cell walls on nutrient bioaccessibility, the release of nutrients from almond tissue has not been studied quantitatively. We have previously quantified the bioaccessibility of carotenoids from vegetables (*23*−*26*) and the release of potentially allergenic proteins from peanuts (*27*) by modeling both the gastric and duodenal lumen. The aim of the present study was to quantify the release of lipid, protein, and vitamin E from almond seeds during in vitro digestion and to better understand the role of almond cell walls in influencing bioaccessibility. A pilot in vivo ileostomy study was also conducted, and microstructural examination used to evaluate the mechanisms involved in nutrient bioaccessibility.

MATERIALS AND METHODS

Almond Products. Food grade almond seeds (*Amygdalus communis* L. var. Nonpareil) used in this study were produced by Stewart & Jasper Orchards (Newman, CA) and kindly provided by the Almond Board of California, as follows: whole natural almonds (NA), whole blanched almonds (BA), and whole finely ground almonds (FG). Industrially obtained FG did not contain skin (coat), and their particle size was 200 μm. NA and BA were carefully cut into cubes of 2 mm dimension and then stored at room temperature in the dark in sealed airtight bags prior to use. The advantages of using 2 mm almond cubes included an easy identification and separation from the effluent during the ileostomy study, together with the semiquantitative way microscopy could be used for determining structural changes, such as lipid release from intact cells. Previous studies demonstrated that masticated almonds gave a bolus containing 90% of particles smaller than 2 mm (*28*). Data from in vivo digested cubes were correlated to our mathematical model of lipid release from almond seeds, the results of which will be presented elsewhere. Defatted finely ground almond plus almond oil (DG) was prepared by extracting 25 g of FG almonds three times with 400 mL of *n*-hexane for 2 h on each occasion. The oil and dried defatted solid were recombined in the original ratio as the FG almonds.

Chemicals and Enzymes. Egg L-α-phosphatidylcholine (PC, lecithin grade 1, 99% purity) was obtained from Lipid Products (South Nutfield, Surrey, U.K.). Porcine gastric mucosa pepsin (activity = 3300 units/mg of protein calculated using hemoglobin as substrate), bovine α-chymotrypsin (activity = 40 units/mg of protein using BTEE as substrate), and porcine trypsin (activity = 13800 units/mg of protein using BAEE as substrate) were obtained from Sigma (Poole, Dorset, U.K.). Lipase for the gastric phase of digestion was a gastric lipase analogue of fungal origin (F-AP15) from Amano Enzyme Inc. Nagoya, Japan. Porcine pancreatic lipase (activity 25600 units/mg of protein), porcine colipase, and bile salts were obtained from Sigma. All other chemicals were of Analar quality.

In Vitro Digestion Study. The protocol was developed to study the in vitro digestion under gastric and duodenal conditions for all four almond products. Each digestion was performed at least four times and the solid material recovered for analysis. Precautions were taken to exclude oxygen from the digestions. This was a prerequisite for obtaining reliable results in the measurement of vitamin E when strictly anaerobic conditions were used, including at the drying stage.

During the development work for this study, NA cubes were chosen as the "baseline" meal. Gastric lipase activity was set so as to digest between 10 and 15% of the total lipid present during the 2 h gastric digestion of the NA, and pepsin activity was set to a physiological concentration. These conditions were chosen to mimic the in vivo situation, where there is typically 10−15% fat digestion in the stomach (*29, 30*). The lipid, protein, and vitamin E contents of the almond starting materials were (mean ± SD) 54.9 ± 0.46%, 25.9 ± 0.24%, and between 23 and 31 mg/100 g, respectively.

Preparation of Phospholipid Vesicles. Phospholipid vesicles were prepared using a modified method as previously described by Moreno et al. (*31*). Solvent was removed from a 0.94 mL aliquot of PC stock solution (63.5 mmol/L in chloroform/methanol), placed into a 50 mL round-bottom flask, and dried under rotary evaporation to make a thin film of phospholipid. Any residual solvent was removed at room temperature under vacuum overnight after three purges with nitrogen. The PC was then suspended in 12.2 mL of warmed saline (0.15 mol/L NaCl, pH 2.5, at 37 °C), and five 2 mm diameter glass balls were added. The flask was flushed with argon and placed in a shaking incubator (170 rpm, 37 °C) for 30 min. The suspension was then sonicated using a sonication probe (Status US 200, Avestin) at 5 °C in a coolant-jacketed vessel for 7 min with a pulsed cycle of 30% full power on for 0.9 s and off for 0.1 s. The suspension of single-shelled liposomes was filtered through a Nalgene 0.22 μm nylon syringe filter to remove any titanium deposited by the sonicator. The liposomes were then covered with a blanket of argon and put back in the shaking incubator for 20 min to equilibrate at 37 °C.

In Vitro Gastric Digestion. An amount of 1.5 g of each almond meal (NA, BA, FG, DG) was suspended in 12.4 mL of acidic saline (150 mM NaCl, pH 2.5) and readjusted to pH 2.5 with HCl as required. The PC vesicle suspension, pepsin, and gastric lipase analogue were then added so that the final concentrations in the aqueous phase were 2.4 mmol/L, 146 units/mL, and 0.56 mg/mL, respectively, and the almond/aqueous phase ratio was 0.12 g/mL. The samples were placed in an orbital shaking incubator (170 rpm, 37 °C) for 2 h. Control "digestions" of the four almond meals (NA, BA, FG, DG) were performed in saline solution (150 mmol/L, pH 2.5) with no enzyme additions.

To assay the effects of the gastric phase digestion, the solid material was recovered by filtration through Whatman no. 1 filter paper. The residues were washed with distilled water and dried to constant weight under nitrogen.

In Vitro Duodenal Digestion. The in vitro gastric digesta of each almond meal were used as starting material for the simulated duodenal digestion. The pH was raised to 6.5 by addition of NaOH and solutions of bile salts, $CaCl_2$, BIS-TRIS, and enzymes in 150 mmol/L NaCl added, so that the final concentrations were as follows: 4 mmol/L sodium taurocholate, 4 mmol/L sodium glycodeoxycholate, 11.7 mmol/L $CaCl_2$, 0.73 mmol/L BIS-TRIS buffer (pH 6.5), 5.9 units/mL α-chymotrypsin, 104 units/mL trypsin, 3.2 μg/mL colipase, and 54 units/mL pancreatic lipase. The final PC concentration was 2.1 mmol/L, and the almond/aqueous phase ratio was 0.11 g/mL (based on the almond weight used at the start of the gastric digestion). Digestion was performed in a shaking incubator (170 rpm, 37 °C) for 1 h, and residues were collected as described above.

Pilot in Vivo Ileostomy Study. The in vivo pilot study was given a favorable ethical approval by the Norfolk Research Ethics Committee (United Kingdom), and volunteers gave written informed consent. Two female volunteers, both at least 10 years postoperative and with no other medical problems, were recruited into the study. The two volunteers were fed NA and BA on separate occasions at least 7 days apart. Overnight fasted volunteers were given 10 g of 2 mm almond cubes (counted and weighed) for breakfast plus a breakfast cereal of choice (no nuts) and 300 mL of fresh semiskimmed milk. At intervals during the consumption of the breakfast cereal and milk, the almond cubes were ingested without chewing. A further 10 g of cubes was ingested without chewing after 4 h, with a choice of sandwiches and nonalcoholic drinks. Volunteers were instructed to make total ileal effluent collections every 2 h for 12 h and then at convenience for a further 12 h.

Analytical Methods. *Lipolysis Kinetics during in Vitro Duodenal Digestion.* Pancreatic lipase kinetics were measured potentiometrically using a Metrohm 718 pH-stat Titrino by titration of released fatty acids with standard 0.05 mol/L NaOH at pH 6.5 and 37 °C from a post in vitro gastric digested almond sample. The extent of lipolysis was calculated from the amount of NaOH required to neutralize the released fatty acids during 60 min of duodenal digestion.

Lipid Content Determination. Original almond materials and digesta residues recovered from in vitro and in vivo studies were analyzed for total lipid. Lipid extraction was performed with a Soxlet automatic Soxtec 2050 extraction using *n*-hexane as solvent. Fatty acids were determined using the method previously described (*32*). Results of lipid content were expressed as a percentage of dry weight.

Total Protein Determination. Original almond materials and solid residues recovered from in vitro and in vivo studies were analyzed for total nitrogen by micro-Kjeldahl according to the AOAC (*33*). Protein was calculated as N × 5.85 and expressed as a percentage dry weight.

Vitamin E Extraction and Determination. The tocopherols are lipophilic and were assumed to be extracted along with the lipid. Vitamin E recovered in almond oil was determined by a conventional HPLC instrument (Shimadzu) equipped with an LC 10 AD *Vp* high-pressure isocratic pump, an SCL-10A *Vp* controllern and an RF-10 A_{XL} fluorescence detector (programmed for excitation at 290 nm and emission at 330 nm). Data acquisition was performed using CLASS VP 5.0 software.

All analyses were performed in triplicate at room temperature using a Cyano column (Restek Ultra Cyano 250 × 4.6 mm, 5 μm particle size). The mobile phase was a mixture of hexane and isopropanol (99:1) at a flow rate of 1 mL/min.

Samples were prepared by dissolving hexane-extracted almond oil (0.1 mL in 1 mL of hexane) and injecting directly onto the HPLC column. Calibration curves and retention times of authentic α-, β-, δ-, and γ-tocopherol standards (Sigma) in hexane were also obtained. The CV (percent) relative to peak areas (three replicates) for the tocopherols present in the almond oils ranged from 0.7 to 10.7%.

Cell Wall Analysis. To assess any changes in the almond cell wall composition during digestion or any effect of blanching, cell wall material (CWM) was prepared from finely ground almonds (FG), finely ground almonds post gastric + duodenal digestion (FG DD), and blanched almonds (BA) using a modified method as previously described (*19*). The alditol acetates were quantified by gas−liquid chromatography, using the method of Blakeney et al. (*34*). Total uronic acids were determined colorimetrically at 580 nm following the method of Blumenkrantz and Asboe-Hansen (*35*).

Microstructural Analysis. The original almond samples (undigested) and samples digested in vitro and collected from ileostomy volunteers were examined by light microscopy (LM) and transmission electron microscopy (TEM) to provide information about the progress of digestion and the physical structure of cell walls post ingestion.

All almond samples were dehydrated in graded acetone or ethanol serial dilutions (i.e., 50, 70, and 90%, by volume, acetone or ethanol and distilled water) for 30 min for each solution and then finally in 100% acetone or ethanol for 30 min (three times). For TEM and LM, the almond samples were infiltrated with Spurr resin, embedded in molds, and polymerized at 60 °C. Sections (1 μm for LM) were cut on a Reichert Ultracut unltramicrotome (Leica Microsystems Ltd., Milton Keynes, U.K.), mounted on glass slides, and stained in 1% (w/v) Toluidine Blue. Ultrathin sections (~70 nm) were cut for TEM and viewed in a JEOL 100CX Mk.II transmission electron microscope (JEOL Ltd., Welwyn Garden City, U.K.).

Statistical Analysis. Differences between in vitro mass losses during digestion were assessed by Student's *t* test. Lipid, protein, and vitamin E losses were assessed by ANOVA followed by Tukey's pairwise comparison. Lipolysis kinetics were analyzed in the same way during the entire time course of fatty acid release. Regression analyses were performed on in vivo mass losses against mean residence time and in vivo lipid against vitamin E losses. Regression values were considered to be significant at $p < 0.05$.

RESULTS

Rate and Extent of Lipase Activity during Duodenal Digestion. Pancreatic lipase kinetics (extent and rates of lipolysis) post simulated gastric digestion were measured potentiometrically by titration of released fatty acids with NaOH under simulated duodenal digestion conditions (**Figure 1**). From the plot of the fatty acids released during digestion of FG, it can be seen that there was no measurable lag time before the onset of lipolysis. After the initial 30 min, the lipolysis rate settled down to a constant over the time course of the experiment. This would indicate that no product inhibition has occurred within the digestion conditions and time period used. Because the digestion conditions were the same for all almond meals, the difference in lipolysis rates between meals would indicate that there is a difference of accessible lipid in the four meal types.

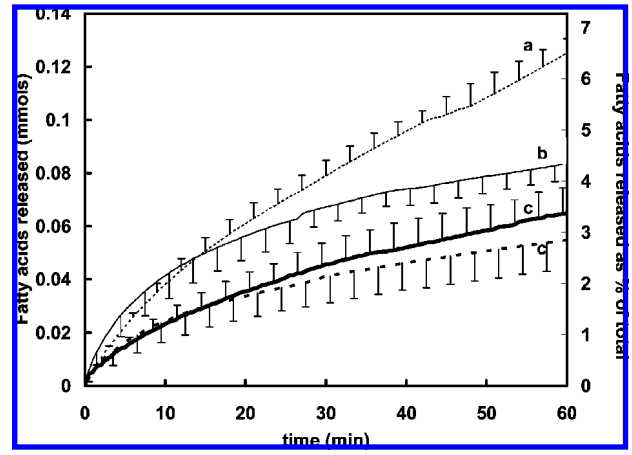

Figure 1. Lipolysis rates measured by pH-STAT under simulated in vitro duodenal conditions from 1.5 g of almonds (initial pregastric digestion weight). Values are the means of duplicate measurements. For clarity, range bars are shown on only one side of the mean values and restricted to 3 min intervals. FG, light dotted line; DG, light continuous line; BA, heavy continuous line; NA, heavy dotted line. Different letters attached to each curve indicate the entire time courses of acid release differed significantly ($p < 0.001$).

Table 1. Mass Losses (Percent) from Almond Meals Due to in Vitro Gastric and Gastric + Duodenal Digestion[a]

almond meal	GD	DD
NA	12.9 ± 0.91	14.0 ± 0.73
BA	14.1 ± 0.66*	15.8 ± 1.17
FG	37.1 ± 0.81**	44.2 ± 0.54
DG	37.4 ± 0.78**	40.1 ± 1.60

[a] Values are the means ± SD, $n = 6$. NA, natural almond cubes; BA, blanched almond cubes; FG, finely ground almond; DG, defatted finely ground almond + almond oil; GD, in vitro gastric digestion; DD, in vitro gastric + duodenal digestion. * and ** show significant differences between gastric and duodenal digestions at $p < 0.01$ and $p < 0.0005$, respectively.

Changes in Mass and Composition of Almond Seed Following Digestion. The loss of mass for all four almond meals, as measured gravimetrically, post in vitro gastric and gastric + duodenal digestion is shown in **Table 1**. Because almond cell walls are not degraded in the upper GI tract (*19*), the mass loss is predominantly related to loss of intracellular components, such as lipid and protein. The simulated gastric digestion step was responsible for the majority of the mass loss in all four almond products, with FG showing the greatest mass loss in both gastric (37%) and gastric + duodenal (44%) conditions. The mass loss for BA cubes was slightly higher than for the NA cubes. The pilot ileostomy study showed a much greater mass loss for the almond cubes (36 and 34% with NA and BA, respectively) compared with the in vitro digestion model. An explanation for this difference could be the longer residence time (average time of 8 h) of the almond cubes in the GI tract of the ileostomists, relative to the in vitro system.

Table 2. Lipid and Vitamin E Losses (Percent) from Almonds Due to in Vitro Gastric and Gastric + Duodenal Digestion[a]

	in vitro gastric digestion			in vitro duodenal digestion		
almond meal	lipid	vitamin E	protein	lipid	vitamin E	protein
NA a	7.6 ± 0.18	10.5 ± 0.02	11.4 ± 0.68	9.9 ± 0.71	16.5 ± 0.06	13.9 ± 0.97
BA a	9.7 ± 0.38	13.2 ± 0.09	12.8 ± 0.77	13.0 ± 0.21	18.9 ± 0.16	14.1 ± 0.32
FG b	31.1 ± 0.25	37.7 ± 0.28	37.9 ± 0.68	39.3 ± 0.12	45.2 ± 0.50	43.8 ± 0.97
DG b	32.1 ± 0.51	37.4 ± 0.30	37.6 ± 0.26	34.0 ± 0.24	39.2 ± 0.32	39.6 ± 0.23

[a] Values are the means (range), $n = 2$. NA, natural almond cubes; BA, blanched almond cubes; FG, finely ground almond; DG, defatted finely ground almond + almond oil. Different letters (a, b) indicate significant difference between almond types for both lipid and vitamin E for in vitro digestions ($p < 0.005$).

Total Lipid Loss during Digestion. Loss of total lipid as a percentage of the original amount present (54.9%) for all four almond meals, post in vitro gastric and gastric + duodenal digestion is shown in **Table 2**. As observed for the total gravimetric losses (**Table 1**), the FG was the most digestible with the highest extent of lipolysis (39%), followed by the DG (34%). Both the whole NA and the BA gave less digestible lipid (10 and 13%, respectively). For all almond meals, the gastric + duodenal digestion produced only a slight increase in lipid loss over that observed in the gastric environment. A higher duodenal solubilization referred to total percent of lipid release was recorded for FG (21%) compared to DG (6%). This effect could be due to differences in the physical form of the lipid presented for digestion: the lipid within the FG was finely dispersed, whereas in the DG the oil had been added back as a bulk lipid phase. When the lipid was present as a bulk phase, the surface area of the oil−water interface at which the lipase acts was likely to be significantly less than that of a lipid phase made up of finely dispersed lipid droplets, and therefore a reduction in lipase activity would be expected. This effect was confirmed by light microscopy (results not shown).

Between 35 and 50% of the original lipid had been lost in vivo. Consistent with the mass losses, substantially higher losses of lipid were generally observed in the in vivo data, relative to the in vitro values.

Lipid Composition. The monounsaturated fatty acids (mainly oleic and palmitoleic acid, 66 and 0.5% of the total, respectively) represented the principal component within almond seeds, followed by the polyunsaturated (mainly linoleic acid, 26% of total) and the saturated (mainly palmitic and stearic acid, 6 and 1% of total, respectively). Only traces of the other fatty acids were detected. No significant differences were observed in the relative proportions of fatty acids composition post in vitro gastric and post in vitro gastric + duodenal digestion for all four types of almond meal investigated, and analogous results were obtained with the in vivo ileostomy samples (results not shown). This probably reflects the limited hydrolysis and the low amounts of saturated fatty acids in the lipase-susceptible positions (*36*).

Vitamin E Loss during Digestion. The total vitamin E of all four almond meals ranged between 23 and 31 mg/100 g, of which 96% was represented by α-tocopherol, 1.3% by β-tocopherol, and the remainder by γ-tocopherol. Losses of vitamin E (percent) from in vitro digested samples are shown in **Table 2**. The loss of vitamin E was shown to be correlated with lipid losses: 45% of vitamin E was lost after simulated gastric and gastric + duodenal digestion of FG, whereas less release was observed with NA and BA cubes. As for lipid digestion, a higher duodenal release referred to the total percentage of vitamin E was recorded for FG (17%) compared to DG (5%). For the in vivo ileostomy study, between 37% (volunteer 2) and 55% (volunteer 1) of the total vitamin E was lost from volunteer samples, indicating vitamin E from almonds is bioaccessible and therefore potentially available for absorption. The percentage loss of total vitamin E was weakly correlated ($r = 0.895$, $p = 0.105$) with the percentage loss of lipid and indicated that vitamin E losses ran ahead of lipid loss.

Protein Loss during Digestion. Losses of total protein, expressed as percentage of original amount for all four almond meals tested post in vitro gastric and gastric + duodenal digestion, are shown in **Table 2**. For the NA and BA cubes digested in the in vitro models, 11 and 13%, respectively, of the protein was lost in the gastric; only slightly more was released into the duodenal environment, giving a total protein loss of 14% for both NA and BA. The FG had the most digestible protein and, in agreement with the lipid data, the gastric digestion removed most of the accessible protein (38%), whereas a slight additional protein loss was seen following the duodenal phase (9% more). Protein loss from representative samples of NA and BA cubes recovered from the ileal effluent was generally higher compared with the in vitro digestion systems and showed a similar trend as for lipid digestion.

Effect of Processing and Digestion on Almond Cell Wall Composition. Sugar composition of the cell wall material (CWM) of BA, FG, and finely ground almonds post duodenal digestion (FG DD) are shown in **Figure 2**. Carbohydrate comprised 65, 62, and 50% of the dry weight of the CWM from FG, FG DD, and BA, respectively. Arabinose, glucose, and galacturonic acid were the major sugars of all the almond meals tested, followed by xylose. Rhamnose and galactose were present in small amounts, and only traces of fucose were detected. The concentrations of different monomeric sugars (expressed as a percentage of total sugar content) including arabinose, glucose, galacturonic acid, and xylose were 39.87, 16.66, 21.21, and 11.96% for FG, respectively. Approximately 9% of the glucose of CWM from FG could be released by hydrolysis with 1 mol/L sulfuric acid alone (results not shown), indicating that the bulk of the CWM glucose was cellulosic in origin. The total amounts of the sugars arabinose, rhamnose, galactose, and galacturonic acid in the sulfuric acid hydrolysis fraction accounted for 81% of the total sugar content. These results indicated that the almond seed cell walls are mainly composed of arabinose-rich polysaccharides, including pectic substances, encasing the cellulose microfibrils. The presence of pectin is consistent with the thick middle lamella at cell wall junctions, previously identified by a TEM study (*19*). No significant changes in the sugar composition of nonstarch polysaccharides (dietary fiber) of almond seeds were observed after in vitro digestion, suggesting that no degradation of the cell wall occurred under these conditions. These results are compatible with previous findings in healthy subjects (*19*), indicating that the cell walls of almond seeds remain largely intact after chewing and digestion, thereby reducing lipid bioaccessibility by hindering the release of lipid available for digestion and access of lipase enzymes into the cells.

Microstructure of Almond Seed Cotyledon. Micrographs of almond seed cubes digested in vivo and in vitro for 3 and 3.5 h, respectively, showed clear evidence of structurally intact

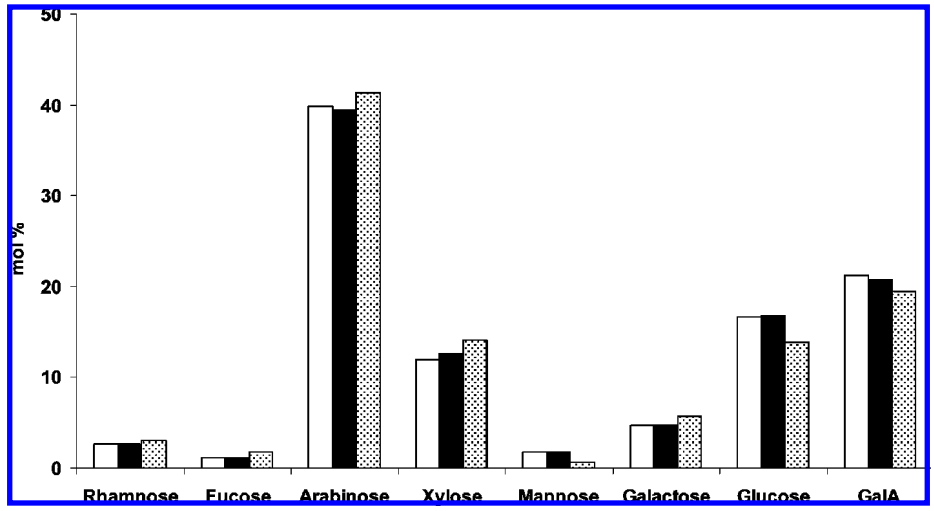

Figure 2. Monosaccharide composition (mol %) of finely ground almonds (white bars), finely ground almonds post duodenal digestion (black bars), and blanched almonds (gray bars). Gal A, galacturonic acid. Values for the total and each sugar concentration are the means of three replicates; the SD of the replicates was always <3%.

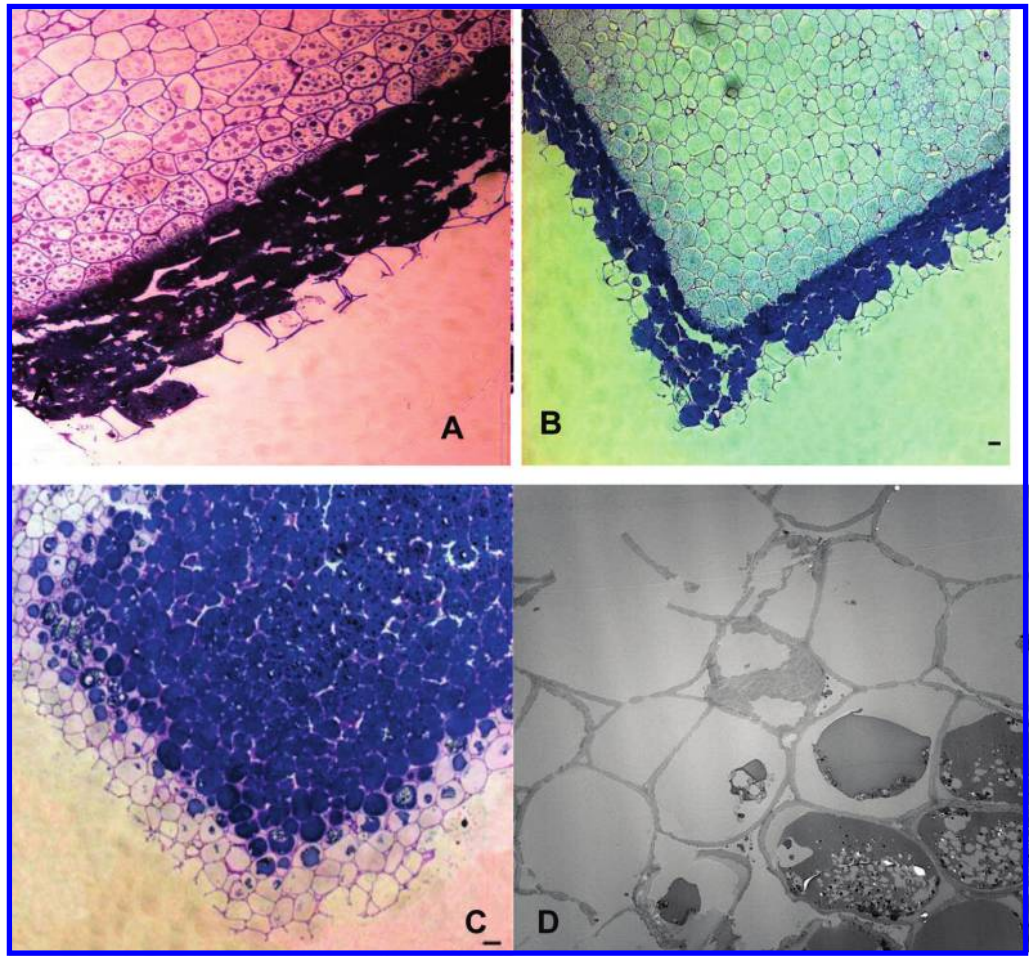

Figure 3. Sections of a 2 mm natural almond cube digested in vitro or in vivo. Light microscopy (LM) section, stained with toluidine blue, shows that after 3 h of simulated gastric + duodenal digestion (**A**) the nutrients of the cells in the first cellular layer (fractured cells) have been digested. The cell walls and intracellular nutrients are still intact in the underlying cells. Similar LM section after 3.5 h in vivo digestion (**B**). LM section after 12 h in vivo digestion (**C**) showed release of nutrients underneath the fractured surface (about three to five layers). Transmission electron microscopy after 12 h in vivo digestion (**D**) showed losses of intracellular contents from intact cells underneath the fractured surface.

cells (i.e., no cell wall rupturing) beneath the fractured layer, which has been created by cutting during cube preparation. In the intact cells, the intracellular nutrients including lipid and protein, showed no signs of digestion (**Figure 3A**,**B**). However, the fractured cell layer at the cut surface of the almond cube showed that the exposed intracellular nutrients had been fully

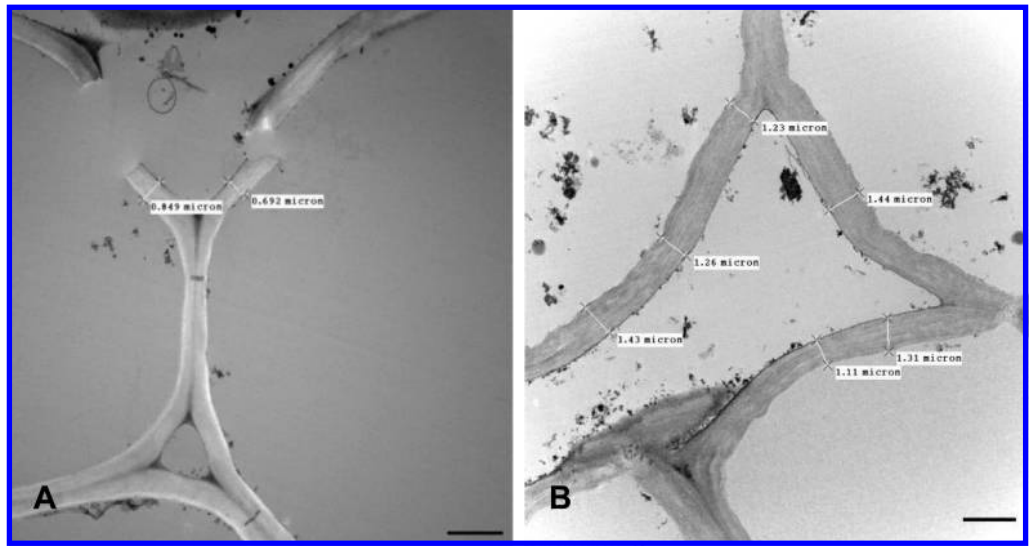

Figure 4. Sections of almond seed tissue digested in vivo. (**A**) Micrograph produced by transmission electron microscopy (TEM) after 2 h digestion showing the size of the cell walls; average cell wall thickness, ~0.6 μm. (**B**) TEM micrograph similar to that seen in panel **A** but after 12 h digestion; average cell wall thickness, ~1.2 μm. Scale bars = 2 μm.

digested. In contrast, sections of almond cubes digested in vivo for 12 h (**Figure 3C**) showed release of nutrients not only in the fractured layer but also in the underlying cells (about three to five layers), where the cell walls had not been ruptured. TEM of sections of almond cubes digested in vivo for 12 h (**Figure 3D**) showed that the cells at the fractured layer and the cells directly underlying these had lost all intracellular content (although these underlying cells appeared to be still intact). The cells directly underlying these empty cells showed that only a small portion of the total cell contents remained, and then a gradient of increasing appearance of cell contents was shown moving into the almond tissue until approximately five cells deep, where the cells appeared to have no intracellular losses.

In all of the micrographs we examined there was no evidence of new fractured layers in addition to those created by cutting, suggesting that the loss of intracellular nutrients from internal cells could only occur by a process of diffusion through the cell walls.

Examination of the cell wall morphology using TEM (**Figure 4**) showed an increase in the average thickness of the cell walls and middle lamella after 12 h of digestion (**Figure 4B**) relative to the 2 h incubation (**Figure 4A**) and to an undigested sample (micrograph not shown). Indeed, the cell wall thickness of the 12 h sample showed a 2-fold increase compared with the 2 h sample and a 6-fold increase relative to the undigested sample. This suggests there has been a significant increase in swelling of the cell walls, which seems to be dependent on incubation time during digestion.

DISCUSSION

The results of our current study confirm our previous observations (*19*) that the almond cell walls (dietary fiber), when physically intact, play primary roles in influencing nutrient bioaccessibility. Intact cell walls act as a physical barrier, encapsulating nutrients and hindering the rate and extent of nutrient release. Hence, only cells that are ruptured by physical means (e.g., chewing or processing) are able to rapidly release nutrients that are available for digestion and absorption. This provides an explanation of why in a number of studies a significant proportion of lipid in almonds and other tree nuts remains undigested and is excreted in feces from human subjects consuming a nut-rich diet (*18, 19*). However, in the present study, we have quantified nutrient bioaccessibility using an in vitro method of gastric and duodenal digestion. Furthermore, our pilot ileostomy study provided indirect evidence of another mechanism that may influence the release of nutrients from almond seed tissue and which is also regulated by the cell walls.

The in vitro digestion model used in this study is a "static" model, as the digestion products are not removed during the time course of the experiment, and during lipolysis the released fatty acids have a potential inhibitory effect on the pancreatic lipase activity. The high fat content of almonds was taken into consideration during the development of the in vitro model to prevent product inhibition. Indeed, the pH-STAT measurements confirm that after 2 h of simulated gastric incubation followed by 1 h of duodenal digestion, there was no indication of product inhibition. Most of the lipid in the almond meals was released in the gastric lumen, whereas much smaller amounts were further released in the duodenal environment. The pattern of losses can be explained by the immediate loss of lipid from the surface of tissue fragments in the gastric environment, after which the integrity of the almond cell walls reduces the rate of release of intracellular lipid. As previously suggested (*19*), lipid from ruptured almond cells is more accessible for emulsification and subsequent digestion by the gastric and pancreatic lipase in the stomach and small intestine, respectively (*37*). This would suggest an easy solubilization of this lipid from ruptured cells, whereas the intracellular lipid retained by the cell wall barrier in intact cells cannot be assessed. There is no evidence for changes in the chemical composition of the almond cell walls before and after digestion (**Figure 2**). Nevertheless, microstructural examination of the almond tissue provides indirect evidence to suggest that intracellular nutrients in unfractured cells are also bioaccessible, albeit released at a much slower rate. The LM micrographs of almond tissue collected from the ileostomy volunteers 12 h after ingestion (**Figure 3C**) showed greater nutrient loss compared with samples collected after 2 h (**Figure 3B**). Nutrients were released after 12 h of in vivo digestion not only from the fractured surface but also from the underlying cells with intact cell walls. TEM micrographs of sections of almond cubes digested in vivo for 12 h (**Figure 3D**) showed that intact cells underlying the fractured layer had lost their

intracellular content. Also, the TEM micrographs of the almond seed tissue provided evidence of substantial cell wall swelling and suggested that the diffusion of enzymes, bile salts, and products of lipid and protein digestion are facilitated by this process (**Figure 4**).

A characteristic of lipases acting on water-insoluble substrates is their "activation" by interfaces (*38*−*40*). A feature often observed during lipolysis is a lag time prior to the establishment of lipolysis. This has been interpreted as the time taken for the lipase molecule to penetrate the emulsion interface and is related to the composition of the interface and the presence of inhibitory factors, such as surface active proteins and protein fragments. It is therefore of interest that we observed no lag times in our kinetic studies of lipolysis, showing that almonds have lipid−aqueous phase interfaces that are potentially easily assessed. Therefore, the low amount of lipid release must be due to the relatively impermeable cell walls that sequester the lipids away from the lipases. In this context it is of interest that the loss of protein during in vitro digestion in the gastric and gastric + duodenal environments followed the same pattern as lipid loss (**Table 2**). A mechanism of slow penetration of the enzymes to their substrates would explain this behavior, and we are currently investigating this process further.

Almonds represent one of the richest dietary sources of vitamin E, which may contribute to the health benefits associated with almond consumption, such as a reduction in the risk of CHD and certain cancers (*41*). Despite the nutritional importance of vitamin E, the factors that influence its bioavailability are poorly understood (*42, 43*), although recent data indicated that its absorption is correlated with the amount of fat in a meal and the food matrix (*44*). Therefore, efforts to improve vitamin E status through dietary means, including almonds, may be warranted. In the present study we showed that about 45% of the total vitamin E content was bioaccessible from finely ground almonds and therefore potentially available for absorption. Vitamin E losses are related to and preceded lipid release, suggesting almonds are a rich source of vitamin E, although providing fewer calories than calculated from nutrient databases.

In summary, the results of the present study indicate that restricted amounts of lipids and proteins are bioaccessible during digestion of almonds and bioaccessibility is improved by increasing the number of fractured cells by mechanical processing and therefore potentially by prolonged chewing. There is a temporal release of almond nutrients during digestion, which is regulated by the cell walls, and the slow release during gut transit could be a result of leaching through swollen cell walls.

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