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Impact of Fatty Acyl Composition and Quantity of Triglycerides on Bioaccessibility of Dietary Carotenoids

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A carotenoid-rich salad meal with varying amounts and types of triglycerides (TG) was digested using simulated gastric and small intestinal conditions. Xanthophylls (lutein and zeaxanthin) and carotenes (α-carotene, β-carotene, and lycopene) in chyme and micelle fraction were quantified to determine digestive stability and efficiency of micellarization (bioaccessibility). Micellarization of lutein (+zeaxanthin) exceeded that of α- and β-carotenes, which was greater than that of lycopene for all test conditions. Micellarization of carotenes, but not lutein (+zeaxanthin), was enhanced ($P < 0.05$) by addition of TG (2.5% v/w) to the meal and was dependent on fatty acyl chain length in structured TG (c18:1 > c8:0 > c4:0). The degree of unsaturation of c18 fatty acyl chains in TG added to the salad purée did not significantly alter the efficiency of micellarization of carotenoids. Relatively low amounts of triolein and canola oil (0.5–1%) were required for maximum micellarization of carotenes, but more oil (~2.5%) was required when TG with medium chain saturated fatty acyl groups (e.g., trioctanoin and coconut oil) was added to the salad. Uptake of lutein and β-carotene by Caco-2 cells also was examined by exposing cells to micelles generated during the simulated digestion of salad purée with either triolein or trioctanoin. Cell accumulation of β-carotene was independent of fatty acyl composition of micelles, whereas lutein uptake was slightly, but significantly, increased from samples with digested triolein compared to trioctanoin. The results show that the *in vitro* transfer of α-carotene, β-carotene, and lycopene from chyme to mixed micelles during digestion requires minimal (0.5–1%) lipid content in the meal and is affected by the length of fatty acyl chains but not the degree of unsaturation in TG. In contrast, fatty acyl chain length has limited if any impact on carotenoid uptake by small intestinal epithelial cells. These data suggest that the amount of TG in a typical meal does not limit the bioaccessibility of carotenoids.

KEYWORDS: Carotenoid; bioaccessibility; triglyceride; salad; *in vitro* digestion; Caco-2 cells

INTRODUCTION

Many of the hundreds of carotenoids in nature are C40 compounds with 10 to 11 conjugated double bonds. Some of these compounds are often classified as carotenes (e.g., lycopene, LYC; α-carotene, AC; and β-carotene, BC) and others as xanthophylls (e.g., lutein, LUT; zeaxanthin, ZEA; and β-cryptoxanthin). Xanthophylls are more hydrophilic than carotenes because of the presence of hydroxyl and keto groups on one or both ionone rings. The ability to quench singlet oxygen and reactive oxygen species, serve as precursors for vitamin A, modulate transcription of target genes, and enhance communication between adjacent cells via gap junctions are among the many reported biological activities of carotenoids and their metabolites (*1–5*). In order to carry out such activities in tissues, dietary carotenoids must be bioavailable, i.e., transferred from the food matrix to target tissues. The bioavailability of carotenoids from a meal can be affected by numerous factors, including chemical speciation, food matrix, styles of processing and cooking, interactions with other dietary compounds such as fiber, lipids, phytosterols and other carotenoids during digestion and absorption, gut health, nutritional status, and other conditions that affect digestion and absorption processes (*6*).

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Among dietary factors that influence bioavailability, co-ingestion of lipids appears to have the greatest impact on the absorption of ingested carotenoids. It was originally reported that 3–5 g of fat was required for the absorption of BC from a meal (*7, 8*). Brown et al. recently reported that absorption of AC, BC, and LYC from salad with dressing containing 28 g of canola oil was greater than that containing only 6 g of canola oil (*9*). Moreover, subjects who ingested a carotenoid-rich salad meal with either avocado oil alone or avocado fruit containing an equivalent amount of oil had greater plasma response to ingested carotenes compared to subjects who ingested a fat-free, carotenoid-rich salad meal (*10*). Several human studies also have shown that the fatty acyl composition of triglycerides (TG) can affect carotenoid absorption. Co-ingestion of a BC-supplement with beef tallow increased the level of BC in plasma TG-rich lipoproteins in human subjects to a greater extent than that with an equivalent amount of sunflower oil (*11*). Also, the amounts of BC and retinyl esters in the chylomicron fraction of plasma were greater when TG containing long chain fatty acyl groups was co-ingested with a salad meal by human subjects compared to TG with medium chain fatty acyl groups (*12*). Moreover, micellar oleic and eicosapentaenoic acid, but not linoleic acid, have been reported to enhance BC absorption and its cleavage into retinol in rats (*13*).

The absorption of carotenoids, like that of other lipophilic compounds, is characterized by a series of processes that include the release from the food matrix and dissolution in oil droplets, lipase- and bile salt-dependent partitioning into mixed micelles for delivery to the apical mucosal surface for uptake by enterocytes, and incorporation of the intact compound or its esterified cleavage products into chylomicrons for secretion into lymph (*14*). Although the amount and physicochemical characteristics of dietary lipids likely affect one or more of these steps, specific mechanisms for the dietary lipid–carotenoid interaction have received limited attention.

We report here the results from investigations on the influence of the amount and structure of TG on the bioaccessibility of carotenoids. Bioaccessibility refers to the efficiency of transfer of a carotenoid from food matrix to mixed micelles during digestion (*15*). Incorporation into mixed micelles is required for lipophilic compounds to be transported across the unstirred water layer for uptake by small intestinal epithelial cells. The first specific aim was to evaluate the influence of chain length and degree of unsaturation of acyl groups in TG and amount of TG on the micellarization of carotenoids from a typical western salad using simulated gastric and small intestinal digestion. The usefulness of this model as a positive predictor of carotenoid bioavailability *in vivo* has been established (*15*). The second aim was to examine the influence of TG fatty acyl chain length (c8:0 vs. c18:1) on intestinal cell uptake of carotenoids from micelles generated during simulated digestion.

MATERIALS AND METHODS

Chemicals. Unless indicated, all other chemicals were purchased from either Fisher Scientific (Norcross, GA) or Sigma-Aldrich (St Louis, MO). Lutein standard was a gift from Dr. Zoraida DeFreitas (Kemin Foods Inc., Des Moines, IA). TG with either *cis*-9, *trans*-11 conjugated linoleic acids (CLA) or *trans*-10, *cis*-12 CLA were purchased from Cognis (Cincinnati, OH). Dietary oils were purchased from a local grocery store, and fatty acyl composition of the dietary oils was determined by saponification followed by fatty acyl methylation in the presence of 20% 1,1,3,3-tetramethylguanidine (TMG) in methanol (v/v) and analysis by Gas Chromatography (GC) (*16*). As expected, predominant fatty acids were c18:2 (68.0%) and c18:1 (15.6%) in safflower oil, c18:1 (58.9%) and c18:2 (19.7%) in canola oil, and c12:0 (46.8%), c14:0 (19.8%), and c16:0 (10.3%) in coconut oil. Caco-2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Preparation of Test Meals. A western-type salad was prepared to examine the effects of chain length and degree of unsaturation of fatty acyl groups in TG on the micellarization of carotenoids during *in vitro* digestion. The salad contained the following carotenoid-rich vegetables and fruits: 20% spinach; 35% tomato; 25% carrot; 10% romaine lettuce; and 10% orange pepper (w/w). The salad was homogenized with a KitchenAid hand mixer until puréed at room temperature. Aliquots were transferred to screw cap polypropylene tubes and stored under nitrogen gas at −80 °C. Carotenoid content of the frozen salad purée was determined monthly, and it remained stable (i.e., equivalent to fresh prepared purée) for at least 6 months. Lipids were extracted from 1 g of salad purée with 2 mL of chloroform and 1 mL of methanol, saponified, and methylated with 20% TMG in methanol to determine fatty acyl composition by GC (*16, 17*). Fatty acids accounted for 0.1% of the wet weight of salad purée, and c18:2 (29%) and c18:3 (37%) were predominant.

To investigate the effect of fatty acyl chain length on the uptake of carotenoids presented in micelles by Caco-2 cells, the test meal contained a mixture of puréed spinach (0.6 g) and carrot (1.0 g) with 2.5% (v/w) of either triolein or trioctanoin. These vegetables were used instead of the salad to generate micelles containing higher amounts of LUT, BC, and AC for the experiment with Caco-2 cells described below.

***In Vitro* Digestion.** The general procedure for the simulated gastric and small intestinal digestion was based on the method described previously with modifications (*18, 19*). The salad purée (3 g) with indicated quantities of test lipids was subjected to simulated digestion. The purée was diluted with 27 mL of saline (120 mM sodium chloride). The pH for gastric digestion was adjusted to 3.0 ± 0.1 instead of 2.0 ± 0.1, 40 mg of pepsin in 2 mL of HCl (0.1 M) was added, and the total volume was increased to 40 mL with saline. For small intestinal digestion, porcine pancreatic lipase (Sigma, L3126) was added along with porcine pancreatin and bile extract to facilitate greater hydrolysis of triglycerides in the test meal. Final concentrations of the bile extract, pancreatin, and pancreatic lipase for small intestinal digestion (50 mL total volume) were 2.4, 0.4, and 0.2 mg/mL, respectively.

After completing the simulated gastric and small intestinal phases of digestion, an aliquot (10 mL) of chyme was centrifuged (Type 50 Ti rotor, Beckman Model L7-65) at 167,000*g* at 4 °C for 20 min to separate the aqueous fraction containing mixed micelles from residual solids and oil droplets. The aqueous fraction was filtered (Millex GP, 33 mm diameter, 0.22 μm pore size, Millipore) to prepare the micelle fraction. Chyme and the micelle fraction were blanketed with nitrogen gas, stored at −20 °C and analyzed within a week.

Uptake of Carotenoids by Caco-2 Human Intestinal Cells. Details regarding the maintenance of cultures of Caco-2 cells (HTB-37, ATCC) have been described previously (*19*). One modification for the present experiment was the substitution of HEPES (15 mmol/L) by PIPES as a buffer in the DMEM to maintain the pH of the medium at 6.8 instead of 7.4. For experiments, cultures of Caco-2 at passages 25–26 were seeded in six well dishes (Falcon, Morristown, TN) at 5×10^4 cells/cm^2 and were used between 11 to 13 days post-confluency.

Mixed micelles with carotenoids for addition to cultures of Caco-2 cells were generated by digestion of a purée of spinach (0.6 g) and carrot (1 g) containing either triolein or trioctanoin (2.5%, v/w). The micelle fraction contained LUT, AC, and BC. To prepare the filtered aqueous fraction with different concentrations of these three carotenoids but similar amounts of micelles, the micelles produced by digesting the salad purée with either triolein or trioctanoin were diluted 4:0, 3:1, 1:1, 1:3, and 0:4 (v/v) with carotenoid-free micelles generated by digesting the same lipid without the salad purée. Appropriately diluted micelle fractions then were mixed with 3 volumes of basal DMEM with L-glutamine (2 mmol/L, GIBCO) and nonessential amino acids (10 mL/L, GIBCO). Caco-2 cells were harvested after 4 h of exposure to media with the various concentrations of carotenoids as reported by Chitchumroonchokchai et al. (*19*). Microscopic examination prior to harvesting suggested

that monolayers remained intact and gross cell morphology was not affected by test treatments. Similarly, the mean cell protein content per well was independent of treatment.

Extraction of Carotenoids from Salad Purée, Chyme and Micellar Fraction. The salad purée was thawed, homogenized, and 2 g purée mixed with 100 μg of apo-8′-β-carotenal (internal standard) in 1 mL of petroleum ether, 2 g of Celite, 1 g of calcium carbonate, and 6 mL of methanol. Then 50 mL of cold (4 °C) petroleum ether/acetone (3:1) was added and the mixture homogenized for 60 s (Ultra-Turrax tissumizer, Janke & Kunkel, IKA Equipments). The homogenate was filtered (2.5 μm pore size, Whatman, grade 42), and the residue was repeatedly extracted 3 to 4 times until both residue and filtrate were colorless. After extraction, combined filtrates were saponified in the dark with 50 mL of 30% (w/v) potassium hydroxide in methanol for 30 min. The organic fraction was washed with 10 mL of deionized water twice and separated from the aqueous fraction using a separatory funnel. The organic fraction was then passed though anhydrous sodium sulfate to adsorb residual water and diluted to 100 mL with petroleum ether. Aliquots (2 mL) were dried under a stream of nitrogen and stored at −20 °C. The recovery of apo-8′-β-carotenal from the purée exceeded 90%.

Carotenoids were also extracted from the chyme and micelle fraction as described previously (*19*). Briefly, 1 μg of apo-8′-β-carotenal in 100 μL of petroleum ether was added to either 2 mL of chyme or micelle fraction as an internal standard, and carotenoids were extracted three times with petroleum ether/acetone (2:1) containing 0.1% (w/v) of butylated hydroxytoluene (BHT). Organic fractions were combined and dried under a stream of nitrogen gas for HPLC analysis. The recovery of apo-8′-β-carotenal from the chyme and micelle fraction exceeded 88%.

Carotenoid Analysis by HPLC. Dried extracts were reconstituted in 1 mL of ethyl acetate/methanol (1:1) and analyzed by HPLC (Waters 2695 separation module with a Waters 2996 photodiode array detector). Carotenoids were separated by using a C18 reverse phase column (Vydac, 201TP54, 4.6mm × 250mm, particle size 5 μm) that was protected by a Nova-Pak C18 guard column (Waters Corporation, Milford, MA). Analytes were eluted from the column with differing proportions of methanol containing 2% water with 1 M ammonium acetate (solvent A) and ethyl acetate (solvent B). The solvent gradient was as follows and passed through the column at a flow rate of 1 mL/min: 0–5min, 100% A; 6–25 min, 100% to 80% A; 25–30 min, 80% to 100% A; 30–35 min, 100% A. Carotenoids were detected at 450 nm. Retention time and absorption spectra of pure standards were used to identify and quantify LUT, ZEA, AC, BC, and LYC. Because the chromatographic procedure failed to completely separate LUT and ZEA, total area of the two peaks were determined, and results are expressed as LUT+ZEA for salad, chyme, and aqueous fractions.

Miscellaneous Assays. Cell protein was determined using bicinchoninic acid assay (BCA; Pierce, Rockford, IL) with bovine serum albumin as a standard.

Statistical Analysis. Statistical analysis was performed using SPSS/Win 14.0. The efficiency of micellarization was calculated for each carotenoid in each meal sample. Data are expressed as the mean ± SEM. Significant differences for effects of amount and type of lipids were tested by one-way ANOVA followed by Tukey's post hoc test. A minimum of 3 observations (n = 3–6) were made to determine whether there were significant differences between groups. The differences were considered significant at $P < 0.05$.

RESULTS

Carotenoid Profile in Salad before and after Simulated Digestion. Puréed salad contained LUT (+ZEA) (2.33 mg/100 g), AC (1.17 mg/100 g), all-*trans* BC (3.83 mg/100 g), 9-*cis* BC (0.33 mg/100 g), and LYC (3.72 mg/100 g). All six carotenoids were detected and quantified in chyme and filtered aqueous (micelle) fraction after simulated digestion. (**Figure 1**) Recovery of carotenoids ranged from 70% to 95%, suggesting relatively high stability during the gastric and small intestinal phases of digestion. The ratio of all-*trans* BC and 9-*cis* BC did not change significantly ($P > 0.05$) in chyme compared to the undigested purée, suggesting limited isomerization. Because the concentration of 9-*cis* BC was quite low in the filtered aqueous fraction, 9-*cis* BC will not be discussed further.

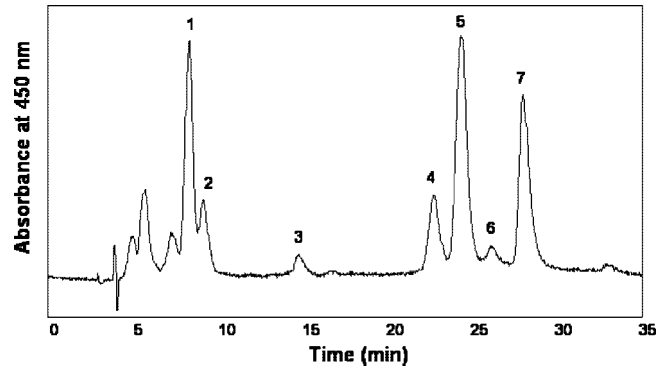

Figure 1. HPLC profile of representative chromatograms of carotenoids from the test salad purée. Carotenoid species were identified by comparing retention times and absorption spectra with the pure carotenoid standards. 1. lutein; 2. zeaxanthin; 3. apo-8′-β-carotenal (internal standard); 4. α-carotene; 5. all-*trans*-β-carotene; 6. 9-*cis*-β-carotene; 7. lycopene.

Effect of TG with Different Fatty Acyl Groups on Micellarization of Carotenoids during Simulated Digestion of Salad Purée. The amount of LUT (+ZEA), AC, BC, and LYC transferred to the filtered aqueous fraction during digestion of purée containing 2.5% (v/w) canola oil was 35.7 ± 0.6%, 13.1 ± 0.2%, 14.3 ± 0.1%, and 4.8 ± 0.1%, respectively. In contrast, the relative amounts of BC, AC, and LYC in the aqueous fraction were only 3.2 ± 0.2%, 1.9 ± 0.3%, and 1.0 ± 0.1%, respectively, when canola oil was not added to the salad purée before digestion. Partitioning of LUT (+ZEA) into the aqueous fraction during the digestion of purée without canola oil was slightly, but significantly ($P < 0.05$), greater (45.0 ± 0.6%) than that with canola oil. Several observations supported the conclusion that carotenoids in the filtered aqueous fraction were present in mixed micelles. First, deletion of pancreatin and pancreatic lipase during the small intestinal phase of the digestion of purée with 2.5% (v/w) canola oil decreased the efficiency of transfer of carotenoids into the filtered aqueous fraction to 16.1 ± 0.4% for LUT (+ZEA) and less than detectable amounts for BC, AC, and LYC. Second, only 2.0 ± 0.1% of LUT (+ZEA) and no detectable BC, AC, and LYC was present in aqueous fraction when bile extract was absent during the small intestinal phase of digestion.

We investigated the effect of fatty acyl chain length and the degree of unsaturation on the micellarization of carotenoids in the salad purée by adding a single TG (2.5%, v/w) with the identical fatty acyl group in *sn*-1, *sn*-2, and *sn*-3 positions. The addition of tributyrin significantly ($P < 0.05$) increased the micellarization of AC and BC, but not LYC, compared to that present in micelles after the digestion of salad purée without TG (**Table 1**). The micellarization of AC and BC was further increased ($P < 0.05$) when either trioctanoin or triolein was added to the salad purée. Partitioning of LYC in micelles during digestion was significantly ($P < 0.05$) increased above that of the control (i.e., no oil) by the addition of trioctanoin to salad purée. Substitution of triolein for trioctanoin further enhanced ($P < 0.05$) the micellarization of LYC. Surprisingly, the addition of all TG except trioctanoin slightly decreased the micellarization of LUT (+ZEA). Because the molecular weights of TG with various acyl groups differ, we also compared the efficiency of micellarization of the carotenoids when the amount of lipids added to the purée was expressed as μmol per gram of salad

Table 1. Chain Length, but Not Degree of Unsaturation of Acyl Groups in TG Affects Micellarization of Carotenoids during *in Vitro* Digestion of Salad*

	efficiency of micellarization (%)			
	lutein (+zeaxanthin)	α-carotene	β-carotene	lycopene
no oil	45.6 ± 0.6^{c}	2.0 ± 0.3^{a}	2.8 ± 0.2^{a}	1.1 ± 0.1^{a}
c4:0	33.6 ± 1.0^{a}	4.9 ± 0.2^{b}	5.3 ± 0.1^{b}	1.4 ± 0.1^{a}
c8:0	$42.0 \pm 1.5^{b,c}$	8.6 ± 0.7^{c}	10.5 ± 0.9^{c}	2.9 ± 0.2^{b}
c18:1	34.8 ± 1.5^{a}	14.9 ± 1.1^{d}	17.7 ± 1.3^{d}	5.2 ± 0.5^{c}
c18:2	34.9 ± 0.4^{a}	15.3 ± 0.7^{d}	18.5 ± 0.3^{d}	5.6 ± 0.6^{c}
c18:3	$38.2 \pm 1.5^{a,b}$	16.6 ± 1.2^{d}	18.3 ± 0.7^{d}	5.0 ± 0.1^{c}

*The salad purée (3 g) was digested *in vitro* with 2.5% (v/w) TG containing indicated fatty acyl groups in all three *sn*-positions of the glycerol backbone as described in Materials and Methods. Data (mean ± SEM) are the relative (%) efficiency of micellarization of indicated carotenoids during *in vitro* digestion of salad purée containing test TG. Each TG was examined in two separate experiments with three independent digestions per experiment ($n = 6$). The presence of different letters as superscripts within a column indicates that the efficiency of micellarization of the carotenoid significantly ($P < 0.05$) differed when the purée was digested without and with the indicated TG.

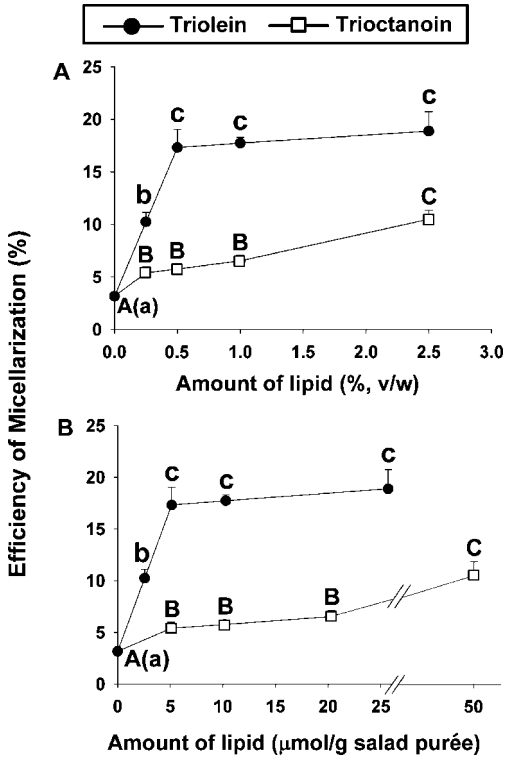

Figure 2. Addition of triolein to salad purée increases micellarization of β-carotene during simulated digestion more efficiently than trioctanoin. The amount of trioctanoin and triolein is expressed as % volume (panel **A**) or μmol (panel **B**) added per gram of wet weight. Data are the mean ± SEM from three independent digestions for each amount of TG added to salad purée ($n = 3$). The different letters above the error bars indicate that the mean efficiency of micellarization of BC differs significantly ($P < 0.05$) in response to the amount of added TG, with uppercase letters indicating trioctanoin and lower case letters indicating triolein.

purée. Micellarization of BC (**Figure 2**), like that of AC and LYC (data not shown), significantly increased with increasing acyl chain length of TG added to salad purée when expressed as either percentage (v/w) or μmol per gram of salad purée.

Micellarization of carotenoids was not significantly altered by the degree of unsaturation of the c18 fatty acids (c18:1, c18:2, and c18:3) (**Table 1**). Similarly, the position of the double bond of the fatty acid in TG did not affect the efficiency of the micellarization

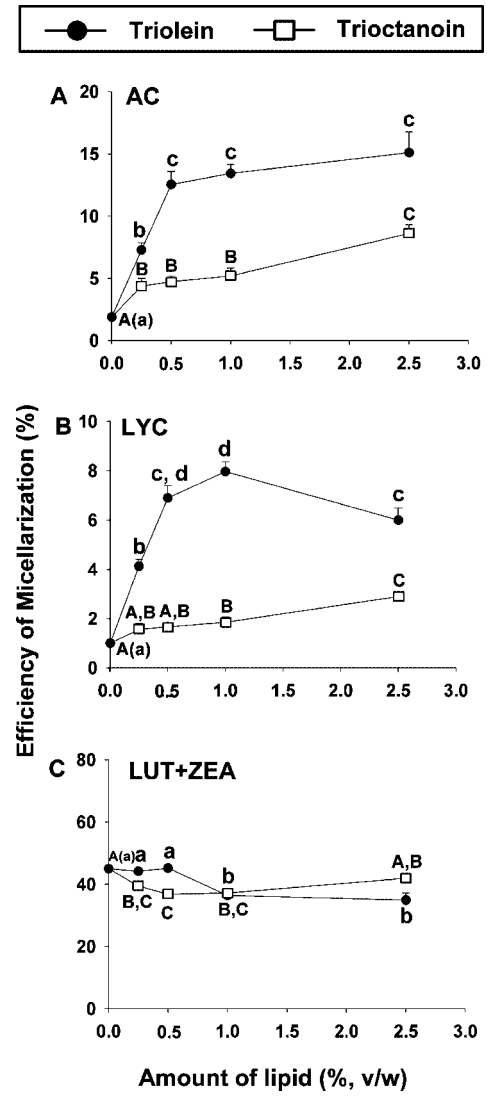

Figure 3. Fatty acyl chain length and amount of TG affect micellarization of α-carotene (AC) and lycopene (LYC) but not lutein + zeaxanthin (LUT+ZEA). Indicated amounts of either triolein or trioctanoin were added to the salad prior to simulated digestion. Micellarization of AC and LYC from the purée containing triolein was significantly higher than that from purée containing the equivalent volume of trioctanoin ($P < 0.05$). Data are expressed as the mean ± SEM from three independent observations ($n = 3$). The different letters above the error bars indicate that the mean percentages of the specified carotenoid micellarization differ significantly ($P < 0.05$) in response to the amounts of lipid added, with uppercase letters indicating trioctanoin and lowercase letters indicating triolein.

of carotenoids during simulated digestion. Partitioning of carotenoids into micelles during the digestion of salad purée containing 2.5% (v/w) TG as either 9-*cis*, 11-*trans* CLA, or 10-*trans*, 12-*cis* CLA was not significantly ($P > 0.05$) different from the purée containing either triolein, trilinolein, or trilinolenin (data not shown).

Different amounts (0.0 to 2.5%, v/w) of trioctanoin and triolein were added to purée that was digested to determine the quantity of TG required for the maximum micellarization of carotenoids. Micellarization of BC and AC almost attained maximum (17.3 ± 3.4%, **Figure 2A**) and (12.5 ± 2.1%, **Figure 3**A) amounts when purée contained as low as 0.5% (v/w) triolein. Micellarization of LYC was maximum (8.0 ± 0.8%, **Figure 3**B) during the digestion of purée with 1.0% (v/w) triolein. In contrast, 2.5% (v/w) trioctanoin was required for

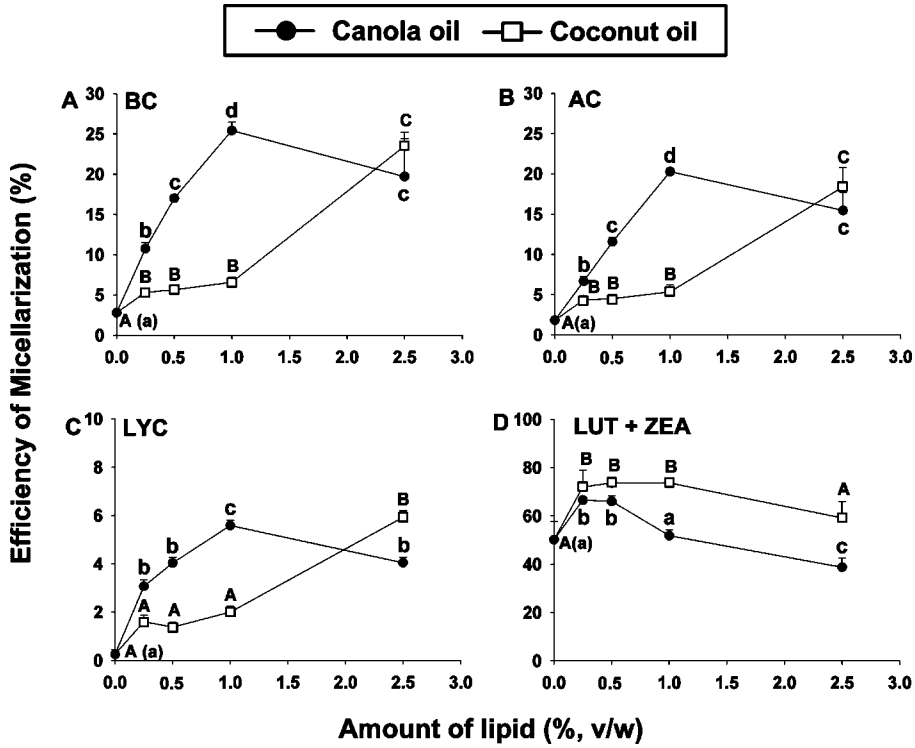

Figure 4. Maximum micellarization of carotenoids during simulated digestion of salad is dependent on the type and amount of dietary oils. Salad purée was digested after the addition of the indicated concentration of either canola or coconut oil (v/w). Micellarization of BC, AC, and LYC during the digestion of salad containing 0.25 to 1% of canola oil was significantly greater than that from salad containing the equivalent volume of coconut oil. Data are expressed as the mean ± SEM from three replicate experiments ($n = 3$). The different letters above or under the error bars indicate that the mean percent of micellarization of the indicated carotenoid differs significantly in response to the concentration of lipid added, with uppercase letters denoting coconut oil and lowercase letters denoting canola oil ($P < 0.05$).

maximum micellarization of BC (10.5 ± 2.1%, **Figure 2A**), AC (8.6 ± 1.7%, **Figure 3A**), and LYC (2.9 ± 0.4%, **Figure 3B**) during digestion. The extent of micellarization at all levels of trioctanoin was significantly ($P < 0.05$) lower than that of samples containing an equivalent level of triolein in purée (**Figures 2** and **3**). Micellarization of LUT (+ZEA) during small intestinal digestion ranged from 38.4% to 45.0% at all concentrations of triolein and trioctanoin added to the salad purée (**Figure 3C**).

The effects of several commercial dietary oils on the micellarization of carotenoids were comparable to that reported above for structured lipids with similar fatty acyl groups. The addition of as little as 0.25% (v/w) canola oil to salad purée significantly ($P < 0.05$) increased the micellarization of carotenes (**Figure 4**). Greater amounts of canola oil further increased the efficiency of micellarization of BC, AC, and LYC to 25.4 ± 1.8%, 20.3 ± 0.8%, and 5.6 ± 0.4%, respectively. Micellarization of the carotenes slightly decreased when the concentration of canola oil increased from 1.0 to 2.5% (v/w). The presence of intact oil droplets on the surface of centrifuged digesta suggested incomplete hydrolysis of a higher volume of TG. Micellarization of carotenes during digestion was not significantly different when 0.25–1.0% (v/w) coconut oil was added to the salad purée but increased markedly when the salad purée contained 2.5% (v/w) coconut oil. We also digested the salad purée with 2.5% (v/w) safflower oil to determine if increasing the degree of fatty acyl unsaturation affected the micellarization of carotenoids during digestion. Micellarization of LUT(+ZEA), BC, AC, and LYC was 33.9 ± 0.5%, 17.6 ± 1.2%, 17.3 ± 1.0%, and 4.5 ± 0.4%, respectively, and not significantly ($P > 0.05$) different from the micellarization of the four carotenoids when the same amount of canola oil was added to the salad purée.

Effect of Acyl Chain Length on Uptake of Micellar Carotenoids by Caco-2 Cells. Cellular uptake of carotenoids was examined by incubating monolayers of Caco-2 cells with serum free DMEM containing micelles generated during the simulated digestion of spinach (0.6 g) and carrot (1.0 g) purée containing 2.5% (v/w) triolein or trioctanoin. The concentrations of carotenoids in the medium were varied by diluting the filtered aqueous fraction with vacant (i.e., carotenoid free) mixed micelles generated during simulated digestion of the test TG only. The highest concentrations of micellarized LUT, BC, and AC in the medium were 0.18 μM, 0.10 μM and 0.06 μM, respectively, which are within the range present in the small intestine after a meal (*20*). The ratio of all-*trans* to 9-*cis* isomers of BC in the test media was not altered during the 4 h of incubation of the medium with micellar carotenoids in the cell culture environment. Accumulation of both LUT and BC increased proportionally with increasing concentrations of the carotenoids in the test media regardless of fatty acyl chain length in the TG added to the purée (**Figure 5**). Cells accumulated 16.8% of LUT from the medium ($y = 0.168x$, $R^2 = 0.93$) during 4 h of incubation with micelles generated from the digested purée with triolein. Cells accumulated slightly, but significantly ($P < 0.05$), less LUT (13.0%) from micelles produced during the digestion of salad containing trioctanoin ($y = 0.130x$, $R^2 = 0.99$). BC uptake was not significantly different ($P > 0.05$) from that of micelles produced during the digestion of salad purée containing triolein (13.5%, $y = 0.135x$, $R^2 = 0.81$) and trioctanoin (12.0%, $y = 0.120x$, $R^2 = 0.91$). Similarly, cells accumulated 13.5% ($R^2 = 0.87$) and 10.7% ($R^2 = 0.81$) AC

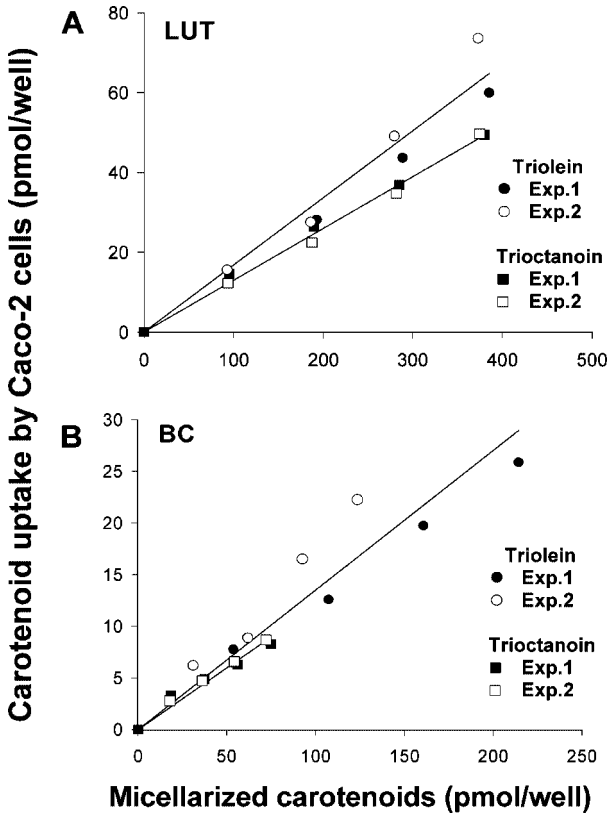

Figure 5. Fatty acyl chain length in dietary TG has minimum impact on the efficiency of the uptake of micellarized lutein (LUT) and β-carotene (BC) by Caco-2 cells. Preparation of micelles added to the cultures of Caco-2 cells is described in Materials and Methods. The highest concentrations of micellarized LUT and BC in media were 0.18 μM and 0.10 μm, respectively. (**A**) The efficiency of LUT uptake by Caco-2 cells from micelles generated during digestion of salad containing triolein (16.8%) is slightly, but significantly ($P < 0.05$), greater than that from micelles produced during the digestion of salad containing trioctanoin (13.0%). (**B**) Efficiency of uptake of BC by Caco-2 cells from micelles generated during digestion of salad with triolein (13.5%) was not significantly different from that with trioctanoin (12.1%). Uptake of LUT and BC by Caco-2 cells was dose-dependent, regardless of TG added to salad. Data in panels **A** and **B** are from two experiments, and each point is the mean of two independent observations. The efficiency of carotenoid uptake for each carotenoid in each treatment is determined by the slope of the regression curves.

from micelles generated during the digestion of purée with triolein and trioctanoin, respectively ($P > 0.05$).

DISCUSSION

The 2005 Dietary Guidelines for Americans by the Departments of Health and Human Services (HHS) and Agriculture (USDA) recommends a reduction of fat intake and encourages the replacement of saturated fatty acids with mono and polyunsaturated fatty acids to reduce the risk of cardiovascular diseases and obesity (*21*). However, the possible impact of such changes in dietary behavior on the bioavailability of ingested fat-soluble vitamins and other health-promoting lipophilic compounds has received limited attention. Because co-ingestion of dietary lipids potently promotes absorption of fat-soluble compounds in foods, optimizing the amount and type of dietary lipids in a meal for maximum bioavailability of fat soluble compounds without increasing calorie intake is important. The present study examined the effects of the amount and type of dietary TG required for maximum micellarization of both hydrocarbon carotenoids and xanthophylls during the simulated digestion of a typical western salad. Micellarization represents a critical process for the delivery or accessibility of carotenoids for absorption. Experiments with structured TG including tributyrin, trioctanoin, and triolein facilitated the evaluation of the impact of chain length and degree of unsaturation on carotenoid micellarization in a controlled fashion using a homogenous lipid system. The model system allows the testing of specific hypotheses prior to more complex experiments with food grade oils and fats (e.g., safflower, canola, and coconut oils).

As expected, the results demonstrate that partitioning of carotenoids into micelles during the simulated digestion of salad was enhanced by the addition of TG to the meal. Efficiency of the micellarization of carotenoids from the salad was influenced by carotenoid structure. Moreover, we confirm that the efficiency of micellarization is inversely proportional to hydrophobicity with LUT (+ZEA) > AC, BC > LYC (*15*, *18*). The results further show that the micellarization of carotenes was dependent on TG acyl chain length but not on the number and position of double bonds. Relatively low amounts (approximately 0.5–1.0%, v/w) of triolein and canola oil were required for maximum micellarization of carotenes, whereas greater quantities were required (approximately 2.5%, v/w) when TG contained medium chain length saturated fatty acyl groups, for example, trioctanoin and coconut oil. While the ability of octanoic acid to form micelle-like structures has been demonstrated (*22*), the extent to which digestion products of tributyrin and trioctanoin partition in mixed lipid micelles in this model is unknown. Therefore, observed increases in apparent micellarization of carotenoids could be due to the direct enhancement of micellarization or another unknown mechanism facilitating the solubilization of carotenoids in the aqueous fraction.

Surprisingly, a decrease in micellarization of LUT+(ZEA) was observed with higher lipid levels (**Table 1**; **Figures 3** and **4**). While statistically significant, the slight decrease at higher lipid levels is likely an artifact of the static nature of the digestion model which did not completely hydrolyze at the highest amounts of lipids tested (2.5%). The residual TG may have solubilized some of the released xanthophylls, reducing the amount partitioned into micelles. While this appears to be an artifact of the model, the overall efficient micellarization of LUT+(ZEA) (~40–50%) without added lipid suggest that only minimal dietary lipid is required for effective solubilization of these polar carotenoids. However, lipid profile and quantity may play a more significant role in subsequent absorptive steps for xanthophylls. For example, the efficiency of LUT uptake by Caco-2 cells was slightly, although significantly, higher from that of micelles generated during the digestion of salad with triolein compared to trioctanoin, while the uptake of BC was not affected by length or unsaturation of fatty acyl chains in added TG.

Numerous factors that influence carotenoid bioavailability have been reviewed elsewhere (*1*, *6*). Dietary fat is required for the efficient absorption of carotenoids and may affect carotenoid bioaccessibility in several ways. First, dietary lipids provide a lipophilic sink to facilitate the transfer of carotenoids from the food matrix to the oil droplets during the gastric phase of digestion. Second, dietary lipids stimulate the secretion of pancreatic lipases and bile salts. The latter are emulsifiers that disrupt large oil droplets to form smaller droplets in which TG and other components are efficiently hydrolyzed by lipases (*23*). Third, the hydrolytic products of dietary lipids may modify

physiochemical characteristics of micelles (*24*, *25*), which possibly enhance the re-partitioning of carotenoids into micelles. Because the standard *in vitro* digestion process is static with fixed amounts of bile salts and pancreatic enzymes, insights regarding the impact of various amounts and types of lipid on each of these steps are unknown. Nevertheless, the simulated digestion model has been used to estimate the bioaccessibility of LUT, ZEA, and all-*trans* and *cis* isomers of BC (*15*, *18*, *19*, *26*–*28*). Borel and associates recently reported that micellarization during the simulated digestion of vegetables and fruits provided a valid estimate of the bioaccessibility of carotenoids *in vivo*. Bioaccessibility as determined by the *in vitro* digestion model was well correlated with outcomes derived from human studies ($r = 0.90$, $P < 0.05$) (*15*).

In the present study, increased acyl chain length of TG was associated with greater efficiency of micellarization of BC, AC, and LYC. This is consistent with previous studies showing that increased fatty acyl chain length (oleate > octanoate > butyrate) enhanced BC uptake in perfused rat small intestine (*29*) and that longer acyl chain length in phospholipids enhanced micellarization and uptake of BC by Caco-2 cells (*30*). Similarly, BC and retinyl palmitate in plasma chylomicrons were dramatically diminished when human subjects ingested BC along with TG containing medium rather than long chain fatty acyl moieties (*12*). Increased acyl chain length increases the hydrophobicity of products of lipid digestion possibly facilitating carotene transfer from the food matrix. Hydrolytic products with different chain lengths produced during gastric and small intestinal digestion also affect the physiochemical characteristics of mixed micelles, and the longer chain acyl compounds likely enhance the re-partitioning of carotenes into micelles (*25*).

Previous investigators have reported that the degree of unsaturation of fatty acyl groups in dietary oils affect the absorption of carotenoids. Clark et al. observed that lycopene and astaxanthin absorption in rats was greater when orally administered as an emulsion with olive oil compared to corn oil (*31*). Hu et al. showed greater absorption of BC and retinyl palmitate after subjects ingested a high fat meal containing beef tallow (46.9% and 50.5% saturated and mono-unsaturated fatty acyl chains, respectively) instead of sunflower oil (68.9% polyunsaturated fatty acyl groups) (*11*). Our *in vitro* observations reveal that micellarization of carotenoids during the simulated digestion of the salad was not altered by the degree of unsaturation of c18 fatty acids or the position of double bonds. This supports the likelihood that the degree of unsaturation of fatty acyl groups influences post-micellarization processes required for absorption such as delivery of the micellarized carotenoids to enterocytes (*31*) or incorporation and secretion of carotenoids in triacylglycerol-rich lipoproteins (*11*). Fatty acyl chain length and degree of unsaturation influence the physiochemical characteristics of micelles including size (*32*). We observed that BC uptake from mixed micelles generated during simulated digestion of salad containing trioctanoin was as efficient as that from micelles generated during the digestion of the salad containing triolein, suggesting that acyl chain length has minimum impact on the efficiency of cellular uptake of carotenes. LUT uptake was slightly (<20%) higher from micelles generated from digested salad with triolein than that from salad with trioctanoin. This suggests that differences in the properties of micelles resulting from the incorporation of digestion products of trioctanoin compared to triolein in micelles had minimal impact on the uptake of the carotenoids by Caco-2 cells. Similarly, Yonekura et al. reported that BC uptake by Caco-2 cells was not proportional to the size of micelles containing either phosphatidylcholine (PC) or lyso-PC with varying fatty acyl chain length (*30*).

Human studies have reported that the amount of dietary fat required for efficient absorption of carotenoids varies from 2.4 to 40 g per meal (*7*–*10*, *33*). In the present study, we showed that the addition of only 0.5–1.0% (v/w) of triolein or canola oil increased the micellarization of carotenes 5–10-fold, while 2.5% trioctanoin or coconut oil was required to achieve maximum micellarization of carotenes (**Figure 4**). On the basis of the data from the Third National Health and Nutrition Examination Survey (NHANES III), the averages for the consumption of salad and salad dressings are ~40 g/day and 3–4 g/day, respectively (*34*). Regular, reduced-fat, low-fat, and fat-free salad dressings contain 10–20 g, 1–8 g, less than 3 g, and less than 0.5 g of fat per serving, respectively. The amount of TG added in the salad purée in the present study is comparable to the fat content when reduced-fat or low-fat dressing is added to the salad. This suggests that the amount and type of dietary fat may affect both the extent of micellarization of carotenoids during digestion and the synthesis and secretion of chylomicrons containing carotenoids following their uptake from micelles. It is interesting that the incubation of Caco-2 intestinal cells with oleate has been shown to stimulate chylomicron synthesis and secretion to a greater extent than linoleate, linolenate, and palmitoylate (*35*–*37*). Studies are now needed to clarify the effects of type and amounts of fatty acyl groups in foods on carotenoid secretion from human intestinal cells in order to better delineate the observed impact of dietary lipid on carotenoid absorption *in vivo*.

To summarize, the addition of TG to a salad markedly increased the bioaccessibility of carotenes, but not xanthophylls, during *in vitro* digestion. The bioaccessibility of carotenes was enhanced by increased fatty acyl chain length but not degree of unsaturation of TG. The uptake of carotenoids from micelles was minimally affected by the fatty acyl composition of TG added to the salad.

ABBREVIATIONS USED

TG, triglyceride; LUT, lutein; ZEA, zeaxanthin; BC, β-carotene; AC, α-carotene; LYC, lycopene; TMG, 1,1,3,3-tetramethylguanidine; BHT, butylated hydroxytoluene; PC, phosphatidylcholine.

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