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Bioaccessibility of Pro-Vitamin A Carotenoids Is Minimally Affected by Non Pro-Vitamin A Xanthophylls in Maize (*Zea mays* sp.)

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The absorption of some carotenoids has been reported to be decreased by coingestion of relatively high concentrations of other carotenoids. It is unclear if such interactions occur among carotenoids during the digestion of plant foods. Current varieties of maize contain limited amounts of the pro-vitamin A (pro-VA) carotenoids β-carotene (BC) and β-cryptoxanthin (BCX) and relatively higher levels of their oxygenated metabolites lutein (LUT) and zeaxanthin (ZEA). Here, we examined if LUT and ZEA attenuate the bioaccessibility of pro-VA carotenoids at amounts and ratios present in maize. BC incorporation into bile salt mixed micelles during chemical preparation and during simulated small intestinal digestion of carotenoid-enriched oil was slightly increased when the concentration of LUT was sixfold or more greater than BC. Likewise, the efficiency of BC micellarization was slightly increased during simulated small intestinal digestion of white maize porridge supplemented with oil containing ninefold molar excess of LUT to BC. Mean efficiencies of micellarization of BC, BCX, LUT, and ZEA were 16.7, 27.7, 30.3, and 27.9%, respectively, and independent of the ratio of LUT plus ZEA to pro-VA carotenoids during simulated digestion of maize porridge prepared from flours containing 0.4−11.3 μg/g endogenous pro-VA carotenoids. LUT attenuated uptake of BC by differentiated cultures of Caco-2 human cells from medium-containing micelles in a dose-dependent manner with inhibition reaching 35% when the molar ratio of LUT to BC was 13. Taken together, these results suggest that the bioaccessibility of pro-VA carotenoids in maize is likely to be minimally affected by the relative levels of xanthophylls lacking pro-VA activity present in cultivars of maize.

KEYWORDS: Pro-vitamin A carotenoids; in vitro digestion; Caco-2 cells; maize; biofortification

INTRODUCTION

Carotenoids are a family of lipid-soluble plant pigments that participate in the light-harvesting process for photosynthesis, protect against photo-oxidative reactions, and attract insects and birds required for dissemination of pollen. About 50 of the 600 carotenoids identified in nature are present in the human diet and several of these [i.e., β-carotene (BC), α-carotene (AC), and β-cryptoxanthin (BCX)] are the primary sources of vitamin A (VA) for populations consuming plant-based diets. Ingested carotenoids and their bioactive metabolites must be transferred across the intestinal barrier to the lymph and ultimately to target tissues to mediate VA-dependent functions that include vision, cellular differentiation, growth, reproduction, and host defense (*1*).

The absorption of carotenoids is influenced by other dietary components in the meal (*2*). For example, fat and digestible fibers enhance and inhibit, respectively, the bioavailability of carotenoids. Some, but not all, studies suggest that the presence of other carotenoids in a meal may also adversely affect the bioavailability of pro-VA carotenoids (*3*). High and Day (*4*) were the first to observe an interaction between LUT and BC when they fed VA-deficient rats diets containing 15 μg of BC and 15−500 μg of LUT. After 12 days, hepatic VA stores were increased and decreased in animals ingesting diets with 3 and >10 times more LUT than BC, respectively. The possibility that carotenoids interact during one or more preabsorptive processes was supported by the observation that coadministration of either 15 mg of LUT or lycopene (LYC) in combination with 15 mg of BC to adult men reduced BC absorption by 34 and 10%, respectively, as compared to BC supplementation administered alone (*5*). In contrast, BC absorption was similar in men fed 15 mg of BC in carrots or carrots coingested with either 15 mg of LYC in tomatoes or 15 mg of LUT in spinach. Kostic et al. also reported that serum BC was similar when healthy adult volunteers ingested a meal with BC supplement (0.5 μmol/kg body weight) alone or with a second supplement containing an equivalent amount of LUT (*6*).

The absorption of carotenoids from the meal requires a series of processes that include its initial transfer from the food matrix

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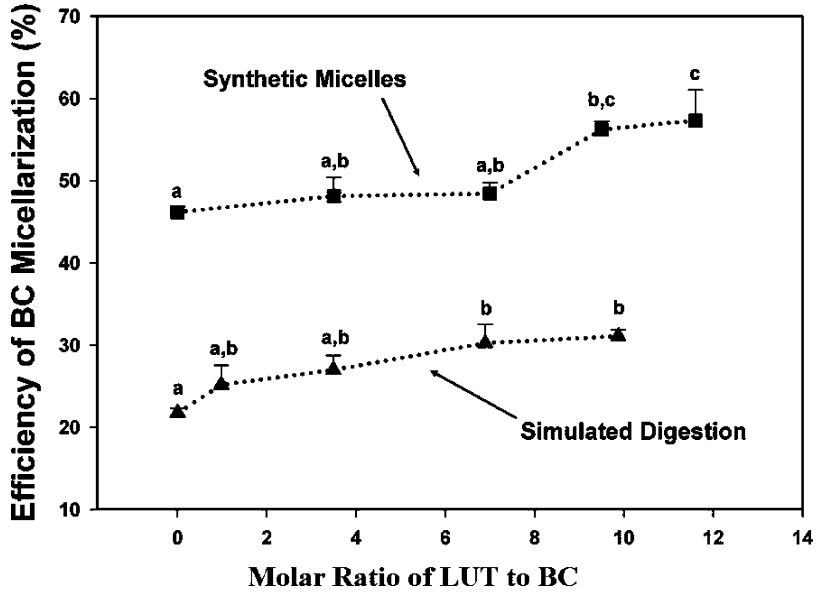

Figure 1. Elevated LUT enhances the efficiency of incorporation of BC into synthetic micelles (■) and micellarization of BC during simulated digestion of white maize porridge supplemented with carotenoid-enriched oil (▲). Data are means ± SEMs ($n = 6$).

Table 1. Concentrations of Carotenoids in Maize Flour Prepared from Test Cultivars[a]

	average carotenoid concn (μg/g maize flour)				ratio of non-pro-VA to pro-VA carotenoids
maize flour	LUT	ZEA	BCX	BC	
white	0.06 ± 0.005	0.05 ± 0.001	ND**	0.04 ± 0.001	2.8
yellow	9.7 ± 0.33	3.8 ± 0.12	1.3 ± 0.09	0.7 ± 0.08	7.0
light orange	10.0 ± 0.29	8.1 ± 0.21	2.8 ± 0.07	4.9 ± 0.09	2.4
dark orange	13.2 ± 0.23	7.9 ± 0.51	2.4 ± 0.05	9.0 ± 0.12	1.9

[a] Data are means ± SEMs ($n = 6$). **ND, not detected.

to oil droplets in the gastrointestinal lumen, partitioning of the pigments into mixed micelles for delivery to absorptive epithelial cells during the small intestinal phase of digestion, and the uptake and incorporation of the pigments into chylomicrons secreted into lymph. Interactions between carotenoids during several of these preabsorptive processes are supported by studies with in vitro model systems. Tyssandier et al. (*7*) reported that addition of either LUT or LYC to an oil emulsion containing an equivalent amount of BC inhibited transfer of BC to mixed micelles during incubation with digestive enzymes and bile salts. It was suggested that the localization of oxycarotenoids near the surface of lipid droplets might decrease the transfer of hydrocarbon carotenoids located within the core of the lipid droplet to micelles (*8*). Transfer of carotenoids across the brush border membrane of absorptive cells occurs by a facilitated process involving scavenger receptor class B type I (SR-BI) (*9*−*11*). During et al. reported that BC uptake from Tween micelles by Caco-2 human intestinal cells was not affected by the presence of as much as five times molar excess LUT when carotenoid concentrations in medium were within the range expected after ingesting a meal (*9*). However, LUT was reported to decrease the intestinal cleavage of BC as assessed by the content of retinyl ester in the triglyceride-rich fraction following ingestion of 15 mg of BC alone as compared with coingestion of BC and an equivalent amount of the xanthophyll (*7*).

Efforts are ongoing to screen maize germplasma to identify cultivars containing increased pro-VA contents for conventional breeding with varieties that possess favorable agronomic traits to generate high-yielding crops enriched in pro-VA carotenoids (*12, 13*). Current varieties of maize generally contain relatively high amounts of LUT and ZEA (carotenoids lacking pro-VA activity) and low amounts of the pro-VA carotenoids BC and BCX.

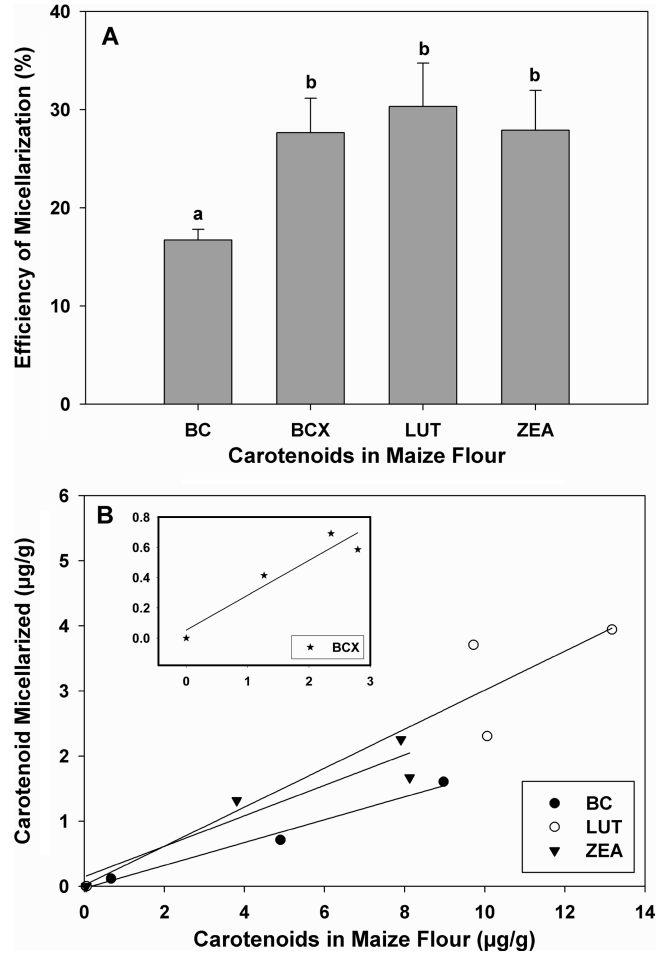

Figure 2. Efficiency of micellarization of xanthophylls is greater than BC during simulated digestion of maize porridge (**A**) and directly proportional to the concentrations of the carotenoids in flour prepared from different cultivars (**B**). Data are means ± SEMs ($n = 6$). Significantly different means ($P < 0.05$) are designated by the presence of different letters above standard error bars.

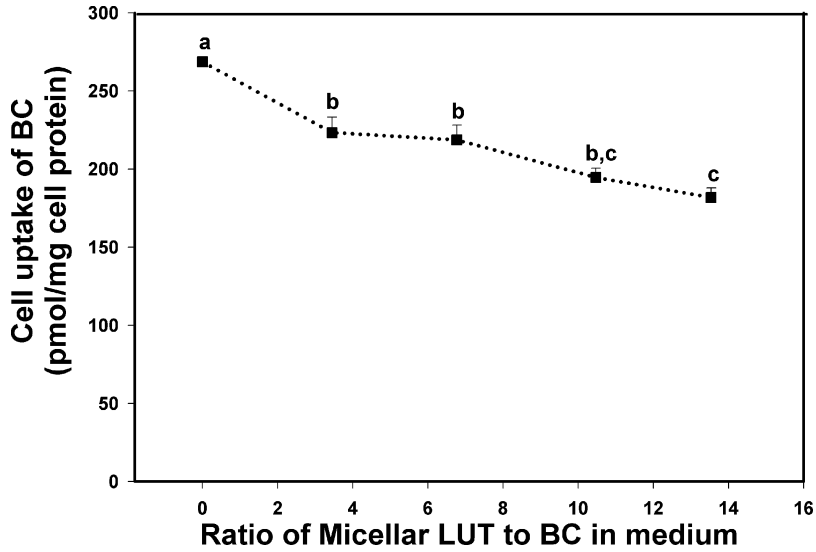

Figure 3. LUT decreases BC uptake by Caco-2 cells when the molar ratio of micellar LUT to BC is >3. The concentration of BC in medium was 2.0 ± 0.10 μmol/L. Data are means ± SEMs ($n = 6$). Significantly different means ($P < 0.05$) are designated by the presence of different letters above standard error bars.

Cultivars selected for increased content of pro-VA carotenoids are expected to also contain increased concentrations of oxygenated carotenoids lacking pro-VA activity, for example, LUT and ZEA. A primary goal of biofortification of maize and other staple plant foods is to provide individuals with adequate amounts of pro-VA carotenoids; the potential effects of LUT and ZEA on the bioavailability of BC and BCX merit examination. Recently, Howe and Tanumihardjo (*14*) reported that high BC maize maintains adequate VA status in Mongolian gerbils. However, this animal, unlike humans, does not absorb xanthophylls (*15*). The basis for this species-specific difference is unknown, and possible interactions between pro-VA and non-pro-VA carotenoids may be confounded in the gerbil model. Therefore, the objective of this study was to examine potential interactions between the carotenoids in maize during the formation of micelles, the physiologic vehicle for delivery of these compounds to the absorptive cell surface, and during uptake of carotenoids by intestinal cells. Specific attention was given to evaluating carotenoid interactions at the ratios of concentrations of these carotenoids present in varieties of maize.

MATERIALS AND METHODS

Chemicals and Supplies. Unless otherwise indicated, all supplies and chemicals were purchased from Sigma-Aldrich and Fisher Scientific.

Preparation of Synthetic Micelles Containing BC and LUT. Aliquots of stock solutions of monoolein, phosphatidylcholine (PC), lyso-PC, α-tocopherol, LUT, and BC in chloroform were combined in a 25 mL glass vial as previously described (*16*). After removal of solvent under a stream of nitrogen at 25 °C, Dulbecco's modified Eagle's medium containing 0.8 mmol/L glycodeoxycholic acid, 0.45 mmol/L taurodeoxycholic acid, 0.75 mmol/L taurocholic acid, and 0.6 mmol/L sodium oleate was added, and the mixture was sonicated in a bath at 25 °C for 30 min. The solution was filter sterilized (0.22 μm pores) to remove insoluble material and microbial contamination. Carotenoids in the micelle fraction (aqueous) were extracted and analyzed by high-performance liquid chromatography (HPLC).

Maize Cultivars and Preparation of Porridge. Cultivars of maize (white, yellow, light orange, and dark orange) were forwarded by Dr. Torbert Rocheford (U. Illinois, Urbana−Champaign) to Dr. Sherry Tanumihardjo (U. Wisconsin, Madison) who prepared flour as reported elsewhere (*14*). Porridge was prepared from maize flour according to the procedure used for a human trial by Dr. Wendy S. White (personal communication, Iowa State University). Briefly, maize flour (20 g) was mixed with DI water (65 mL) and heated with mixing in a Teflon-coated pan at 95 °C for 12 min. The porridge was cooled for 10 min at room temperature before storing in 50 mL polypropylene screw-cap tubes under nitrogen gas at −80 °C until analysis.

Preparation of Carotenoid-Rich Oil. Known amounts of BC alone, LUT alone, or BC with varying amounts of LUT in hexane and 2.3 mg of PC in chloroform were added to 11 mL glass vials followed by 1 mL of extra light olive oil. Mixtures were placed in an ultrasonic bath at room temperature for 30 min with several changes of water to maintain the temperature at <30 °C. The organic solvent was then evaporated under a stream of nitrogen gas at 25 °C.

In Vitro Digestion. Maize porridge (3 g) was subjected to simulated oral, gastric, and small intestinal digestion as described by Thakkar et al. (*17*) with inclusion of carboxyl ester lipase (CEL, 50 U/reaction) during simulated small intestinal digestion to hydrolyze any carotenoid esters in maize (*18*).

Uptake of Carotenoids by Caco-2 Cells. The Caco-2 human intestinal cell line (HTB37, American Type Culture Collection; Manassas, VA) was maintained as previously described (*17*). Differentiated cultures of Caco-2 cells (passages 22−25; 11−13 days postconfluency) were incubated in medium containing synthetic micelles loaded with BC and LUT at ratios indicated in the Results. After 4 h, the medium was removed and monolayers were washed with 2 mL of ice-cold phosphate-buffered saline (PBS), pH 6.8, containing 2 g/L albumin before two additional washes with ice-cold PBS only to remove residual carotenoids adhered to the cell surface. Cells were scraped into 1 mL of cold PBS, pelleted by centrifugation, and stored under nitrogen at −20 °C for a maximum of 48 h before analysis of carotenoids. The protein content of sonicated cells was determined by the bicinchoninic acid (BCA) method (Pierce; Rockford, IL).

Extraction of Carotenoids from Maize Flour and Porridge. The extraction procedure was adapted from Howe and Tanumihardjo (*19*). Briefly, carotenoids were released from dried maize flour (0.6 g) and porridge (0.6 g) by adding 6 mL of ethanol, mixing by vortex (30 s), and heating at 85 °C for 5 min. Potassium hydroxide (500 μL, 80% w/v in water) was added to the mixture to saponify interfering oils. Samples were vortexed for 30 s and returned to the 85 °C bath for 10 min with additional vortexing after 5 min. Samples were immediately placed in ice bath, and 3 mL ice cold deionized water was added for rapid cooling. After adding β-apo-8′-carotenal (2 μg in hexane as recovery standard), carotenoids were extracted into 3 mL of hexane, and the procedure was repeated three times for complete extraction. The pooled hexane fraction was then washed with 3 mL of ice cold DI water. The top hexane layer was transferred to a new vial, and residual carotenoids were extracted from the aqueous layer twice with 3 mL of

hexane two additional times. Hexane was evaporated from the pooled fraction at room temperature under a stream of nitrogen gas, and the film was reconstituted in 1 mL of mobile phase for HPLC analysis.

Extraction and Analysis of Carotenoids. Procedures for the extraction and HPLC analysis of carotenoids in digested samples, aqueous fraction, media, and cells have been described in detail elsewhere (*17*).

Statistical Analysis of Data. Six samples of maize flour were independently extracted and analyzed to determine profile and quantity of carotenoids. Aliquots of separate solutions containing pure BC and varying amounts of LUT were added to three reaction tubes to investigate incorporation of carotenoids into synthetic micelles, and the experiment was repeated once ($n = 6$). A minimum of three independent simulated digestions of test preparations was performed for each experiment, and each experiment was repeated at least once for a minimum of six observations ($n \geq 6$). Preparations of carotenoids in synthetic micelles were added to a minimum of five ($n \geq 5-8$) wells of differentiated Caco-2 cells to examine uptake of the pigments. All data were expressed as means ± standard errors of the mean (SEMs). Statistical analysis was performed using SPSS Release 14.0 for Windows (SPSS Inc., Chicago, IL). Means were compared using one-way analysis of variance followed by Fisher's protected least significant difference for pair wise comparison. The differences were designated as significant at $P < 0.05$.

RESULTS

Effect of LUT on Incorporation BC into Micelles. Approximately 46.2 ± 0.7% of BC added to a vial (212 nmol) was incorporated into synthetic micelles in the absence of LUT (**Figure 1**). Micellarization of BC significantly ($P < 0.05$) increased when the concentration of LUT was 10 and 12 times greater than BC. The efficiency of incorporation of LUT into synthetic micelles was 96.5−98.9% and independent of both the presence of BC and the amount of LUT.

The efficiency of transfer of BC from oil added to white maize porridge into mixed bile salt micelles during simulated oral, gastric, and small intestinal digestion was 22 ± 0.6%. The presence of LUT concentrations as great as seven times that of BC in the oil did not significantly alter the partitioning of BC in micelles formed during in vitro digestion (**Figure 1**). However, the efficiency of micellarization of BC was slightly, but significantly ($P < 0.05$), increased during simulated digestion when the ratio of LUT to BC in oil added to white maize porridge was 7 (i.e., high LUT enhanced the efficiency of BC micellarization). The efficiency of transfer of LUT to micelles during simulated digestion was not affected by the presence of BC and independent of the amount of LUT in the oil added to maize porridge (range, 88.5−93.5%).

Digestive Stability and Efficiency of Micellarization of Carotenoids during Simulated Digestion of Porridge Prepared from Maize Flour. *All-trans* isomers of LUT, ZEA, BCX, and BC were present in flour prepared from yellow, light orange, and dark orange maize cultivars (**Table 1**). AC was identified but not quantified due to a low signal-to-noise ratio. Additional peaks with spectral characteristic of *cis* isomers of LUT or ZEA and epoxy carotenoids also were present, although a lack of pure standards prevented definitive identification of specific isomers. The visual intensity of pigmentation in maize flour was proportional to the total carotenoid content and particularly that of BC in the dark orange sample, which was approximately six times that in yellow maize flour (**Table 1**). The ratio of xanthophylls (LUT + ZEA) to pro-VA carotenoids (BC + BCX) decreased from a maximum of 7.0 in flour from yellow maize to 1.9 in dark orange flour.

Digestive Stability and Efficiency of Micellarization of Carotenoids during Simulated Digestion of Porridges Prepared from Maize Flour. The recovery of the four carotenoids of interest in porridge after simulated oral, gastric, and small intestinal digestion exceeded 90% for the xanthophylls, 88% for BC, and 79% for BCX. The mean efficiency of micellarization during small intestinal digestion of porridge prepared from yellow, light orange, and dark orange maize flour was 28−30% for LUT, ZEA, and BCX, but only 16.7% for BC ($P < 0.05$; **Figure 2A**). The quantities of pro-VA carotenoids, LUT, and ZEA in micelles after small intestinal digestion were highly correlated ($R^2 > 0.88$; $P < 0.05$) with the amount in the maize flour (**Figure 2B**).

Effect of LUT on BC Uptake by Caco-2 Cells. Caco-2 cells accumulated 269 ± 3 pmol BC/mg cell protein (14.1 ± 0.3% of BC in medium) from synthetic micelles during the 4 h incubation. Accumulation of BC significantly ($P < 0.05$) decreased in a concentration-dependent manner from when medium also contained three or more times greater concentration of LUT than the pro-VA carotenoid (**Figure 3**). When the ratio of LUT to BC in medium was 14, cellular accumulation of BC was only 68% that in medium without LUT.

DISCUSSION

More than 50 years ago, High and Day reported increased and decreased concentrations of VA in livers of rats fed 30 μg BC/day in combination with less than and more than 30 μg LUT/day, respectively (*4*). It was unclear if the observed interaction between the administered carotenoids occurred during preabsorptive or postabsorptive processes or both. Tyssandier et al. reported that coadministration of LUT as either supplement (24 mg) or in cooked spinach (12 mg) inhibited BC absorption from tomato puree in adult women (*20*). Similarly, coadministration of LUT (7.5 mg) and BC (15 mg) was reported to significantly decrease the absorption of BC as compared to subjects fed BC alone (*21*). Such data demonstrated preabsorptive interactions between xanthophylls and carotenes and supported the possibility that chronic intake of relatively high amounts of xanthophylls may adversely affect VA status. However, Davis and colleagues reported that large variation in the concentrations of LUT and ZEA in maize flour used to prepare test diets for Mongolian gerbils had minimal impact on bioefficacy of pro-VA carotenoids (*22*). Another study conducted by the same group of investigators suggested that human subjects fed yellow carrots containing LUT and BC for 7 days significantly increased the serum LUT concentration without decreasing the serum BC concentration (*23*). The experimental design did not provide insights about postprandial absorption of the carotenoids. In the present study, we used several in vitro methods to assess potential preabsorptive interactions between pro-VA carotenoids (BC + BCX) and the most abundant xanthophylls in maize using amounts and ratios of these carotenoids present in cultivars.

We first prepared mixed micelles containing products of lipid digestion, bile salts, and various ratios of LUT to BC. At LUT to BC molar ratios of 10 and higher, LUT enhanced BC incorporation into micelles. We next examined if this surprising observation also occurred when oil enriched with BC alone or with varying amounts of LUT was added to white maize porridge subjected to simulated oral, gastric, and small intestinal digestion. The efficiency of BC micellarization significantly increased when the molar ratio of LUT to BC was ≥7. In contrast, Borel and associates (*7*) reported that LUT markedly inhibited transfer of equimolar BC from lipid droplets to mixed micelles in a model simulating small intestinal digestion. It is noteworthy that micellarization of BC during simulated digestion in the absence of LUT in this model system was quite low

(<1%) and likely contributed to the discrepancy between the two studies. LUT and BC are localized on the surface and in the core of oil droplets, respectively (*8*). BC is retained within the core when lipase-mediated hydrolysis of triglycerides is limited, thus decreasing the likelihood for transfer of the hydrocarbon carotenoid to micelles. We also measured the bioaccessibility of endogenous carotenoids in porridge prepared from maize flours containing different endogenous amounts of the pro-VA carotenoids, LUT and ZEA. The efficiency of micellarization of the xanthophylls LUT, ZEA, and BCX significantly exceeded that of BC as previously reported by our group and others (*16, 17, 24*−*27*). The quantity of pro-VA carotenoids micellarized during the small intestinal phase of digestion was proportional to their content in maize flour and independent of the concentrations of LUT and ZEA.

To study the potential interaction during the uptake of carotenoids across the brush border membrane of absorptive cells, differentiated monolayers of Caco-2 cells were incubated in medium containing different ratios of LUT to BC in mixed bile salt micelles. LUT inhibited cellular accumulation of BC in a dose-dependent manner during the 4 h incubation. Such competitive inhibition is not surprising as transfer of carotenoids across the brush border membrane appears to be protein-mediated requiring the participation of SR-BI (*9*−*11*). Accumulation of LUT was saturable as reported previously for Caco-2 cells and independent of the presence of the relatively low concentration of BC (*10, 28*).

In conclusion, this study examined the possibility that the relatively high molar ratio of LUT + ZEA to pro-VA carotenoids generally present in maize attenuates the bioaccessibility of BC and BCX. Working with concentrations of the pro-VA and nonpro-VA carotenoids that are present in varieties of maize, we found that LUT either did not affect or slightly increased the efficiency of incorporation of BC into synthetic mixed micelles and mixed micelles generated during simulated digestion of white maize porridge supplemented with carotenoid-rich oil. Similarly, the amounts of pro-VA carotenoids (BC + BCX) micellarized during simulated digestion of maize porridge prepared from flours processed from different cultivars of maize were proportional to their content and independent of the concentrations of LUT and ZEA. Uptake of BC from micelles by Caco-2 cells was decreased by LUT in a dose-dependent manner. We speculate that the decrease in the uptake of BC by enterocyte-like cells in the presence of relatively high concentrations of LUT may be offset by the increased efficiency of micellarization of BC and presumably other pro-VA carotenoids at ratios of xanthophylls and pro-VA carotenoids present in maize. Furthermore, our observations also suggest that breeding of varieties of maize to contain higher concentrations of pro-VA (BC, AC, and BCX) carotenoids will increase the quantities of these carotenoids and their cleavage products absorbed. Ingestion of such biofortified varieties is expected to improve VA status of those in developing areas where maize is a primary staple food and VA deficiency remains a public health problem.

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