JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of β-Carotene and Lutein Available from Green Leafy Vegetables by an in Vitro Digestion and Colonic Fermentation Method

JOSÉ SERRANO,† ISABEL GOÑI,*,§ AND FULGENCIO SAURA-CALIXTO#

Universidad Del Valle de Guatemala, Guatemala, Guatemala; Department of Nutrition, Faculty of Pharmacy, Universidad Complutense de Madrid, 28040 Madrid Spain; and Department of Nutrition and Metabolism, Instituto del Frío, CSIC, 28040 Madrid, Spain

Green leafy vegetables (*Spinacea oleracea*, *Cnidoscolus aconitifolius*, and *Solanum americanum*) contain a high amount of β-carotene (27−52 mg/100 g of dry sample) and lutein (140−193 mg/100 g of dry sample). The amount of β-carotene and lutein released from the food matrix by the action of digestive enzymes ranged from 22 to 67% and from 27 to 77%, respectively. There was a significant correlation between the enzymatic release of carotenoids (lutein + β-carotene) and the content of Klason lignin, nonstarch polysaccharides, and resistant protein. The carotenoids released by the in vitro colonic fermentation ranged from 2 to 11%, and part of them (0.251−4.03 mg/100 g of original dry sample) remained intact in the fermentation media and could be potentially absorbed in the colon. A significant part of carotenoids seems to be unavailable in the intestinal tract (16% in *S. oleracea* to 58% in *C. aconitifolius*).

KEYWORDS: β-Carotene; lutein; digestibility; colonic fermentability; *Cnidoscolus aconitifolius*; *Solanum americanum*; *Spinacea oleracea*

INTRODUCTION

Current dietary guidelines to combat chronic diseases, including cancer and coronary disease, recommend increased intake of plant foods, including fruits and vegetables, which are rich sources of carotenoids (*1*, *2*) and other bioactive compounds. As antioxidants, carotenoids are protective agents that inactivate reactive oxygen species and may therefore help to delay or prevent oxidative damage. β-Carotene and lutein are major carotenoids in plant foods, especially in green leafy vegetables. Besides possessing provitamin activity, β-carotene is a potent antioxidant, which scavenges free radicals (*3*) and quenches singlet oxygen. Also, several desirable health-related properties of lutein have been identified: for example, studies have linked the intake of lutein and zeaxanthin to the prevention of age-related macular degeneration (*4*, *5*).

Malnutrition, particularly vitamin A deficiency, is one of the major public health problems in developing countries (*6*). Chaya (*Cnidoscolus aconitifolius*) and macuy (*Solanum americanum*) comprise part of the staple diet and are the main dietary source of green leafy vegetables for the indigenous people of the Yucatan peninsula in Mexico and the Kekchi people of Alta Verapaz in Guatemala (*7*). In Central America, *C. aconitifolius* and *S. americanum* constitute important nutritional sources of protein, provitamin A carotenoids, vitamins (ascorbic acid, niacin, riboflavin, and thiamin), and minerals (calcium, iron, and phosphorus) among populations that cannot afford expensive foods rich in these nutrients.

However, the effectiveness of vegetables as a source of carotenoids has been questioned because studies have shown that the bioavailability of β-carotene and lutein from vegetables is less than previously thought. The relative bioavailability of β-carotene from vegetables as compared to purified β-carotene ranges between 3 and 6% for green leafy vegetables and between 19 and 34% for carrots (*8*). Broccoli and green peas induce a stronger β-carotene response in plasma than whole-leaf and chopped spinach despite containing 10 times less β-carotene (*9*). β-Carotene from fruits is reported to be 2.6−6 times as effective in increasing plasma concentrations of retinol and β-carotene as green leafy vegetables (*10*). There are also differences between the relative bioavailability of β-carotene and lutein (*11*, *12*), lutein bioavailability being higher than that of β-carotene. However, these differences observed in in vivo studies may not reflect the true bioavailability of β-carotene, because part of the absorbed β-carotene is cleaved and converted to retinyl esters before entering the blood stream. Therefore, the capacity of the digestive enzymes to release the carotenoids from the food matrix may be the first step to determine the bioavailability. For a better understanding, the use of in vitro digestion and colonic fermentation methods may gain more

* Address correspondence to this author at the Departamento de Nutrición I, Facultad de Farmacia, Avda. Complutense s/n, Ciudad Universitaria, 28040 Madrid, Spain (telephone +34 91 394 18 12; fax +34 91 394 17 32; e-mail igonic@farm.ucm.es).
† Universidad Del Valle de Guatemala.
§ Universidad Complutense de Madrid.
# Instituto del Frío, CSIC.

10.1021/jf0480142 CCC: $30.25
Published on Web 03/19/2005

insight into the availability of carotenoids from the food matrix and the factors that determine their availability.

Only the carotenoids released from the food matrix by the action of digestive enzymes and intestinal microflora are available in the bowel and are potentially susceptible to becoming bioavailable and thus absorbed through the intestinal barrier. However, the amount of food carotenoids available in the bowel may differ significantly from the food content as estimated by chemical analysis. The aim of this work was to evaluate the availability of carotenoids in both the small and large intestines, using an in vitro digestion and colonic fermentation model. Green leafy vegetables common in the Guatemalan and Spanish diets were chosen. Macuy and chaya are part of the staple diet of the Central American population, and spinach is a significant green leafy vegetable item in the Spanish diet.

MATERIALS AND METHODS

Sample Preparation. Chaya (*Cnidoscolus aconitifolius*) and macuy (*Solanum americanum*) are native mesoamerican leaves that were purchased from a local supplier in Guatemala, Central America. Leaves were cooked at atmospheric pressure in boiling water for 15 min. Cooking water was discarded, and leaves were freeze-dried, homogenized in an electric grinder, and stored at −18 °C. Spinach (*Spinacea oleracea*) was purchased from a local supplier in Madrid, Spain. Raw spinach leaves were freeze-dried, homogenized in an electric grinder, and stored at −18 °C. Only the leaves of the three species were used as sample.

Indigestible Fraction Methodology. The procedure to determine the indigestible fraction was described by Saura-Calixto et al. (*13*). Samples were successively incubated with digestive enzymes to simulate digestion in the small intestine. Briefly, 300 mg of sample was incubated with pepsin (0.2 mL of a 300 mg/mL solution in 0.2 M HCl−KCl buffer, pH 1.5, 40 °C, 1 h, Merck 7190), pancreatin (1 mL of a 5 mg/mL solution in 0,1 M phosphate buffer, pH 7.5, 37 °C, 6 h, Sigma P-1750), lipase (2 mL of a 7 mg/mL solution in 0.1 M phosphate buffer, pH 7.5, 37 °C, 6 h, Sigma L-3126), porcine bile extract (2 mL of a 17.5 mg/mL solution in 0.1 M phosphate buffer, pH 7.5, 37 °C, 6 h, Sigma B-8631), and α-amylase (1 mL of a 120 mg/mL solution in 0.1 M Tris−maleate buffer, pH 6.9, 37 °C, 16 h, Sigma A-3176) in subsequent steps. Then samples were centrifuged (15 min, 3000*g*) and supernatants removed. Residues were washed twice with 5 mL of distilled water and all supernatants combined. Some residues were dried overnight at 105 °C and quantified gravimetrically as the insoluble indigestible fraction; other residues were freeze-dried and stored at −18 °C for carotenoids analysis. Each supernatant was incubated with 100 μL of amyloglucosidase (Roche, 102 857) for 45 min at 60 °C. Supernatants were transferred into dialysis tubes (12000−14000 MWCO; Dialysis Tubing Visking, Medicell International Ltd., London, U.K.), and dialyzed against water for 48 h at 25 °C (water flow = 7 L/h). The soluble indigestible fraction was measured in the dialysis retentate as nonstarch polysaccharides by dinitrosalicylic method (*14*), and some dialyzed supernatants were evaporated to dryness with nitrogen flush at 35 °C in an R-114 Büchy vacuum rotatory evaporator and then stored at −18 °C for carotenoids analysis. The total indigestible fraction is the sum of soluble and insoluble indigestible fractions.

In Vitro Colonic Fermentation. The in vitro fermentation method was described by Goñi and Martín-Carrón (*15*). Male Wistar rats were supplied by the breeding center at the Faculty of Pharmacy (University Complutense Madrid, Spain). Fresh rat cecal contents were used as inoculum. Ceca were removed through abdominal midline incisions and diluted (100 g/L) with anaerobic medium. The inoculum was mixed (10 min) in a Stomacher 80 Lab Blender (Seward Medical, London, U.K.) and filtered (1 mm mesh) before use. Residues from enzymatic treatments were hydrated with fermentation medium (8 mL, 4 °C, 16 h). Two milliliters of inoculum was added and the headspace rinsed with carbon dioxide (1 min). Tubes were placed in a shaking water bath (37 °C, 24 h). Lactulose (Sigma L-7877) was included in the experiment as 100% fermentable substrate. Blanks (cecal content) of fermentation were also included. After incubation time, the pH was measured, and 1 M NaOH was used to stop the fermentation process. Samples were centrifuged (2500*g*, 10 min), and the supernatants and residue were collected and stored at −18 °C for carotenoids analysis.

Carotenoids Extraction. Carotenoids were extracted following the procedure described by Quackenbush (*16*) with some modification introduced in our laboratory. Briefly, freeze-dried samples (30 mg) with 10% w/w sodium sulfate, sodium carbonate, and 2,6-di-*tert*-butylhydroxytoluene (BHT) (Panreac Química S.A., Barcelona, Spain) were incubated (30 min at 50 °C) with dimethyl sulfoxide (2.5 mL, O_2 free). After incubation, methanol (5 mL, O_2 free) was added, and the mixture was vortexed vigorously for 30 s and centrifuged (4200*g*, 3 min); supernatants were collected. Methanol washes were employed until methanol was colorless (absorbance ≤ 0.001); all supernatants were combined. The carotenoids extract was saponified following the method described by Granado et al. (*17*) with some modifications introduced in our laboratory. Briefly, a mixture containing carotenoids methanolic extract (2 mL), diethyl ether stabilized with 6 mg/L of BHT (4 mL) (Panreac Química), and 0.5 mL of saturated KOH in water was slightly shaken and allowed to saponify for 30 min in the dark. After saponification, 5 mL of distilled water was added, and the mixture was vortexed for 15 s and centrifuged (4200*g*, 3 min). The ether layer was collected, and several washes with ether were employed until ether was colorless (absorbance ≤ 0.001).

HPLC β-Carotene and Lutein Determination. The HPLC system was a Hewlett-Packard System Series model 1100 with a photodiode array detector. The column was a 4.6 mm × 250 mm C-18 5 μm Nucleosil 100 (Teknokroma, Barcelona, Spain). A guard column (4 mm × 23 mm) containing the same packing material was installed ahead of the carotenoid column. The solvents were HPLC grade methanol (Panreac, Barcelona, Spain) and ethyl acetate (Panreac). A gradient system was used involving two mobile phases. Mobile phase A was methanol/water (75:25 v/v), and mobile phase B was ethyl acetate. The initial values were 100% of A and 0% of B, to 50% A and 50% B in 10 min, followed by 100% B within 15 min. The flow rate was 1.0 mL/min during the entire run, and the column was kept at room temperature. A 50 μL aliquot was used for injection, and detection was done at 445 and 455 nm. Standard curves of lutein (95%, Extrasynthese, 0306 S, Genay-France) and β-carotene (≥95%, Sigma C-4582) were constructed by plotting HPLC peak absorbance area versus concentration of the carotenoid in the injected sample (*18*).

Klason Lignin and Insoluble Nonstarch Polysaccharides (INSP). The insoluble indigestible fraction was treated with sulfuric acid (12 M H_2SO_4, 20 °C, 3 h; dilution to 1 M H_2SO_4 and refluxing for 2 h), and Klason lignin was determined gravimetrically. For INSP analysis, aliquots were taken from the hydrolysate from Klason lignin analysis, and sugars and uronic acids were determined spectrophotometrically with dinitrosalicylic acid (*14*), which corresponds to the total INSP.

Protein. Protein was determined in triplicate in the original material (total protein) and in the indigestible fraction (indigestible protein), using an automated nitrogen analyzer (FP-2000 Dumas, Leco Corp., St. Joseph, MI). Samples were weighed into large ceramic boats and loaded into the furnace, where they were combusted in a pure oxygen environment. After passing through a thermoelectric cooler to condense water, combustion gases were collected in a volume blast, and a 10 mL aliquot was taken. The gases were scrubbed, and protein was reduced to nitrogen and detected by a thermal conductivity cell. An air blank was carried out and the instrument calibrated with EDTA. Protein was calculated as N × 6.25.

Short-Chain Fatty Acids (SCFAs). The method of Spiller et al. (*19*), slightly modified, was followed. Supernatant (400 μL), internal standard (4-methylvaleric acid, 2 mM) (500 μL), and 50 μL of 12% formic acid were made up to 1 mL with Milli-Q water and centrifuged (4 °C, 7300*g*, 15 min). One microliter of supernatant was injected into a 5890 Hewlett-Packard gas chromatograph equipped with a flame ionization detector and a fused silica column (Carbowax 20 M, 10 m × 0.53 mm i.d.). Nitrogen was the carrier gas at a pressure of 17 kPa. Injector and detector temperatures were 200 °C, and the column temperature was 120 °C (isothermal). SCFAs were identified and quantified by comparison with fatty acids standards.

Table 1. Indigestible Fraction Characterization of Green Leafy Vegetables[a]

	g/kg of dry original sample		
	S. oleracea	*C. aconitifolius*	*S. americanum*
insoluble indigestible fraction	474.4 ± 29.1	656.0 ± 21.3	569.9 ± 9.0
Klason lignin	204.9 ± 29.5	415.9 ± 19.4	308.1 ± 42.5
nonstarch polysaccharides	175.3 ± 13.7	90.8 ± 6.7	113.6 ± 4.2
resistant protein	54.4 ± 2.6	103.9 ± 4.3	83.6 ± 1.5
ash	57.0 ± 1.6	51.4 ± 3.1	43.1 ± 0.2
associated carotenoids	0.342 ± 0.045	1.768 ± 0.075	1.107 ± 0.032
soluble indigestible fraction			
nonstarch polysaccharides	34.7 ± 2.0	39.1 ± 4.3	39.0 ± 1.8
associated carotenoids	0.044 ± 0.001	0.028 ± 0.001	0.012 ± 0.001

[a] Mean ± SEM, $n > 4$.

Statistical Analysis of Data. All data were reported as mean ± standard deviation for at least four replicates in each treatment. Simple regression analysis was performed by the computer program Statgraphics Plus, version 2.1 (Statistical Graphics Corp., Inc., Rockville, MD).

RESULTS AND DISCUSSION

There are two main steps in the methodology proposed to estimate the intestinal carotenoid availability in the gut: (a) isolation of the indigestible fraction and (b) colonic fermentation of the indigestible fraction. The indigestible fraction was previously defined as the part of vegetables that is not digested or absorbed in the small intestine and reaches the colon, where it serves as a substrate for fermentative microflora (*13*). This is a physiological alternative to the common dietary fiber concept (*20*), consisting of dietary fiber and other compounds of proven resistance to the actions of enzymes such as indigestible protein, resistant starch, and other associated bioactive compounds. Analytical conditions for indigestible fraction determination were close to physiological conditions (pH, temperature, incubation times). By analyzing the carotenoid content in the residues and supernatants obtained with this methodology, it was possible to estimate the availability of carotenoids in the small and large intestines.

β-Carotene and lutein represent the two main structural carotenoid groups: carotenes and xanthophylls. In addition, β-carotene and lutein account for >75% of total carotenoid content in green leafy vegetables (*21*).

Small Intestine Availability. The three green leafy vegetables analyzed had a total indigestible fraction (soluble + insoluble) ranging from 51% in *S. oleracea* to 71% in *C. aconitifolius*. The contents of the indigestible fraction of the sample and its major constituents are shown in **Table 1**. The insoluble fraction accounted for >90% of the total indigestible fraction of the samples. The insoluble indigestible fraction contained nonstarch polysaccharides, Klason lignin, resistant protein, and minerals (*13*), whereas the soluble indigestible fraction was formed principally by nonstarch polysaccharides. Nonstarch polysaccharides and Klason lignin represent dietary fiber (*21*).

The authors previously reported the presence of polyphenolic compounds associated with dietary fiber or the indigestible fraction, which significantly affects their bioavailability (*23*). Remarkably, there were significant amounts of carotenoids associated with both the soluble and insoluble indigestible fractions of the samples (**Table 1**). A major part was associated with the insoluble indigestible fraction. These carotenoids remained in the food matrix after the enzymatic treatment. Therefore, they were not available in the small intestine and were not susceptible to absorption.

Table 2. Content and in Vitro Small Intestine Availability of β-Carotene and Lutein in Green Leafy Vegetables[a]

	mg/100 g of dry original sample		
	S. oleracea	*C. aconitifolius*	*S. americanum*
original sample			
β-carotene	26.7 ± 2.4	51.5 ± 1.1	52.1 ± 3.6
lutein	139.7 ± 14.4	192.2 ± 37.1	183.8 ± 25.2
insoluble indigestible fraction			
β-carotene	7.6 ± 1.9	39.8 ± 1.2	34.7 ± 1.2
lutein	31.0 ± 4.1	139.9 ± 7.5	77.2 ± 3.0
soluble indigestible fraction			
β-carotene	0.50 ± 0.01	0.30 ± 0.01	0.30 ± 0.01
lutein	1.4 ± 0.1	0.9 ± 0.1	nd
small intestine availability[b]			
β-carotene	67	22	33
lutein	77	27	58

[a] Mean ± SEM, $n > 4$. [b] [Concentration of carotenoid in original sample − concentration of carotenoid in indigestible fraction]/[concentration of carotenoid in original sample] × 100.

The β-carotene and lutein contents in the original samples and their corresponding indigestible fractions are shown in **Table 2**; lutein was the major carotenoid in the three samples. The native Central American samples contained more lutein and β-carotene than *S. oleracea*.

The amount of β-carotene available in the small intestine ranged from 23% of total *C. aconitifolius* content to 72% in *S. oleracea*. Percentages of the same order were observed for lutein. These data suggest that the chemically analyzed carotenoid content may considerably exceed the amount available in the small intestine.

The carotenoids available in the small intestine are potentially susceptible to becoming available and thus absorbed through the intestinal barrier. Nevertheless, the incorporation of carotenoids into the micelle and uptake of the latter may also affect real carotenoid absorption by the enterocyte, where the interaction with bile acids and dietary fat plays a major role in carotenoid bioavailability. Moreover, the soluble indigestible fraction may interfere with bile salt micelle formation, thus reducing the absorption of carotenoids released from the food matrix (*24*).

The relative bioavailability of purified lutein reported in the literature is much higher than that of β-carotene (*11*). The same was found by Castenmiller et al. (*12*) for the relative bioavailability of lutein and β-carotene from spinach (45 and 5.1% respectively), probably because lutein is less lipophilic than β-carotene. However, these differences may not reflect the true bioavailability, because part of the absorbed β-carotene is cleaved and converted to retinyl esters before entering the bloodstream.

There was significant correlation between small intestine availability of carotenoids (lutein + β-carotene) and content of Klason lignin, nonstarch polysaccharides, and resistant protein (**Figure 1**). Klason lignin, nonstarch polysaccharides, and resistant protein contents may directly affect the intestinal availability of carotenoids, because they can act as a barrier to the action of digestive enzymes and to the release of carotenoids from the food matrix. Moreover, the intactness of the cellular matrix has been reported to be a determiner of carotenoid bioavailability from green leafy vegetables, because carotenoids present in chloroplasts are non-covalently bound to protein and fiber in the thylakoid membrane (*25*).

Large Intestine Availability. Nondigestible compounds (indigestible fraction: e.g., nonstarch polysaccharides, resistant protein, and indigestible carotenoids) in green leafy vegetables

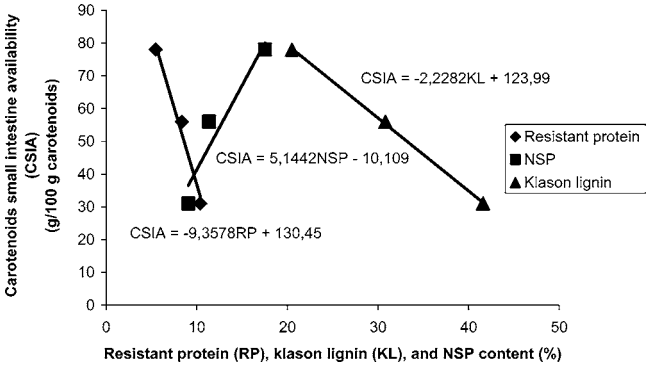

Figure 1. Correlation between carotenoids small intestine availability and protein digestibility, nonstarch polysaccharides (NSP) content, and Klason lignin content. Correlation coefficients (r^2): 0.9805, 0.9994, and 0.9146 for resistant protein, Klason lignin, and NSP, respectively.

Table 3. In Vitro Colonic Fermentation of Nondigestible Compounds of Green Leafy Vegetables[a]

	S. oleracea	*C. aconitifolius*	*S. americanum*
SCFA[b] production (mmol/g of indigestible fraction)	3.5 ± 0.2	4.0 ± 0.1	4.4 ± 0.5
fermentability[c]	25	28	31
molar proportions[d]			
acetate	52.9 ± 3.9	52.0 ± 3.5	52.3 ± 3.5
propionate	42.9 ± 1.4	38.2 ± 4.2	40.4 ± 1.6
butyrate	4.2 ± 0.5	9.8 ± 0.8	7.3 ± 1.1
nonfermented residue (g/100 g of dry original sample)	34.4 ± 3.7	46.8 ± 2.0	40.8 ± 0.6
β-carotene (mg/100 g of dry original sample)	6.0 ± 0.4	39.1 ± 3.8	33.2 ± 2.4
lutein (mg/100 g of dry original sample)	29.5 ± 1.3	124.3 ± 5.8	57.9 ± 2.2

[a] Mean ± SEM, $n > 4$. [b] Short-chain fatty acids. [c] Percentage of fermentability with respect to lactulose = $[SCFA_{green\ leafy\ vegetables}]/[SCFA_{lactulose}] \times 100$. [d] [Production of acetate, propionate, or butyrate]/[total SCFA] × 100.

provide a substrate for the large bowel microflora. They undergo some fermentation, thus producing short-chain fatty acids. The three samples analyzed presented similar levels of fermentability and SCFA production (**Table 3**). The major SCFAs was acetate, followed by propionic acid and butyric acid. Fermentation of green leafy vegetables presented relatively high proportions of propionic acid as compared to other fermentable compounds such as pectin (molar proportion 70:10:20) (*26*). This is interesting in that propionic acid is associated with hypocholesterolemic effects (*27*).

The nonfermented residue obtained after fermentation contains compounds of proven resistance to enzymatic and colonic bacterial degradation, which are probably excreted in the feces; these comprise the unavailable compounds. The amount of unavailable carotenoids associated with nonfermented residue is shown in **Table 3**. **Figure 2** summarizes the percentages of β-carotene and lutein that are not available in the gastrointestinal tract, along with the percentage of small intestine and colon availability. *S. oleracea* had the lowest nonavailable carotenoid content (16%) and is therefore probably a major carotenoid source for the host. The amount of lutein and β-carotene released from the food matrix by the colonic microflora enzymes (colon availability) was low for all samples.

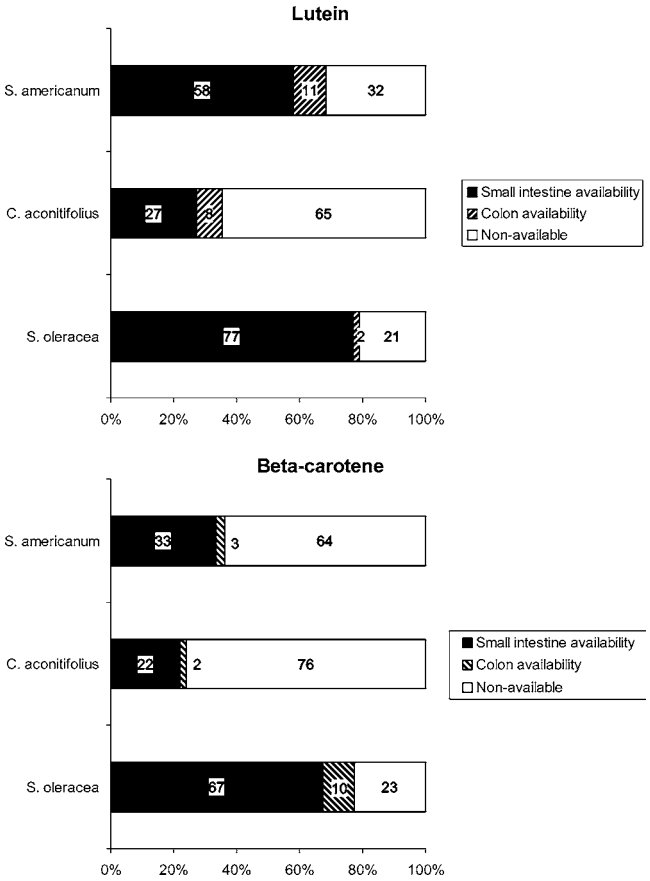

Figure 2. Lutein and β-carotene intestinal availability (mg/100 mg of carotenoids in original sample).

Table 4. Carotenoids Released from the Indigestible Fraction during in Vitro Colonic Fermentation and Not Metabolized by the Colonic Microflora[a]

	mg/100 g of dry original sample		
	S. oleracea	*C. aconitifolius*	*S. americanum*
carotenoid content in the indigestible fraction[b]			
β-carotene	8.1	40.1	35.0
lutein	32.4	140.8	77.2
carotenoids not released by the microflora enzymes (nonfermented residue)			
β-carotene	6.0 ± 0.4	39.1 ± 3.8	33.2 ± 2.4
lutein	29.5 ± 1.3	124.3 ± 5.8	57.9 ± 2.2
carotenoids released by the microflora enzymes[c]			
β-carotene	2.1	1.0	1.8
lutein	2.9	16.5	19.3
carotenoids released by the microflora enzymes but not metabolized by colonic microflora (potentially available for absorption in the colon)			
β-carotene	0.031 ± 0.001	0.23 ± 0.01	0.056 ± 0.003
lutein	0.220 ± 0.017	3.8 ± 0.2	0.87 ± 0.03

[a] Mean ± SEM, $n > 4$. [b] [Carotenoids in soluble indigestible fraction + carotenoids in insoluble indigestible fraction]. [c] [Carotenoids in indigestible fraction − carotenoids in nonfermented residue].

There are no data available in the literature about carotenoid colonic fermentation. Previous studies conducted at our laboratory with carotenoid standards showed high fermentability for β-carotene and zeaxanthin (unpublished observations). Because carotenoid standards were the only substrate for the colonic bacteria, >98% of each standard was degraded to fermentation end-products. Similar results were observed in green leafy vegetables, where a significant part of the carotenoids released from the indigestible fraction in *S. oleracea* and *S. americanum* was used as substrate by the colonic microflora (92 and 96%,

respectively) (**Table 4**). Interestingly, 23% of the carotenoids released from the food matrix by the colonic microflora enzymes in *C. aconitifolius* were not used as a fermentation substrate. Although there is no accurate information or method to evaluate absorption of carotenoids in the colon, some nutrients are available for absorption in the colon. It is probable that part of this 23% of carotenoids in the fermentation media of *C. aconitifolius* is available for absorption in the colon.

In summary, the carotenoid release from the food matrix is a key step in the bioavailability of carotenoids. The content of indigestible fraction, dietary fiber, Klason lignin, and resistant protein in green leafy vegetables affects the enzymatic release of β-carotene and lutein during enzymatic digestion and colonic fermentation. Our results suggest that some carotenoids are available in the small intestine and colon, whereas an appreciable amount is unavailable in the gastrointestinal tract.

LITERATURE CITED

(1) Johnson, E. The role of carotenoids in human health. *Nutr. Clin. Care* **2002**, *5*, 56−65.

(2) Feeney, M. Fruits and the prevention of lifestyle-related diseases. *Clin. Exp. Pharmacol. Physiol.* **2004**, *31*, S11−S13.

(3) Jimenez-Escrig, A.; Jimenez-Jimenez, I.; Sanchez-Moreno, C.; Saura-Calixto, F. Evaluation of free radical scavenging of dietary carotenoids by the stable radical 2,2-diphenyl-1-picryl-hydrazyl. *J. Sci. Food Agric.* **2000**, *80*, 1686−1690.

(4) Seddon, J.; Ajani, U.; Sperduto, R.; Hiller, R.; Blair, N.; Burton, T.; Farber, M.; Gragoudas, E.; Halter, J.; Miller, D.; Yannuzzi, L.; Willet, W. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. *JAMA−J. Am. Med. Assoc.* **1994**, *272*, 1413−1420.

(5) Ribaya-Mercado, J.; Blumberg, J. Lutein and zeaxanthin and their potentiall roles in disease prevention. *J. Am. Coll. Nutr.* **2004**, *23*, 576S−587S.

(6) World Health Organization. *Global Prevalence of Vitamin A Deficiency*; Micronutrient Deficiency Information System Working Paper 2; WHO: Geneva, Switzerland, 1995 (Document WHO/NUT/95.3).

(7) Booth, S.; Bressani, R.; Johns, T. Nutrient content of selected indigenous leafy vegetable consumed by Kekchi people of Alta Verapaz, Guatemala. *J. Food Compos. Anal.* **1992**, *5*, 25−34.

(8) De Pee, S.; West, C. Dietary carotenoids and their role in combating vitamin A deficiency: a review of the literature. *Eur. J. Clin. Nutr.* **1996**, *50* (Suppl. 3), S38−53.

(9) van het Hof, K.; Tijburg, L.; Pietrzik, K.; Weststrate, J. Influence of feeding vegetables on plasma levels of carotenoids, folate and vitamin C. Effect of disruption of the vegetable matrix. *Br. J. Nutr.* **1999**, *82*, 203−212.

(10) De Pee, S.; West, C.; Permaesih, D.; Martuti, S.; Muhilal & Hautvast, J. Orange juice is more effective than are dark-green leafy vegetables in increasing serum concentrations of retinol and β-carotene in school-children in Indonesia. *Am. J. Clin. Nutr.* **1998**, *68*, 1058−1067.

(11) van het Hof, K.; Brouwer, I.; West, C.; Haddeman, E.; Steegers-Theunissen, R.; Van Dusseldorp, M.; Weststrate, J.; Eskes, T.; Hautvast, J. Bioavailability of lutein from vegetables is 5 times higher than that of β-carotene. *Am. J. Clin. Nutr.* **1999**, *70*, 261−268.

(12) Castenmiller, J.; West, C.; Linssen, J.; van het Hof, K.; Voragen, A. The food matrix of spinach is a limiting factor in determining the bioavailability of β-carotene and to a lesser extent of lutein in humans. *J. Nutr.* **1999**, *129*, 349−355.

(13) Saura-Calixto, F.; Garcia-Alonso, A.; Goñi, I.; Bravo, L. In vitro determination of the indigestible fraction in foods: an alternative to dietary fiber analysis. *J. Agric. Food Chem.* **2000**, *48*, 3342−3347.

(14) Englyst, H.; Cummings, J. Improved method for the measurement of dietary fibre as nonstarch polysaccharide in plant foods. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 808−814.

(15) Goñi, I.; Martin-Carron, N. Fermentacion colonica. In *Fibra dietetica en Iberoamerica: Tecnologia y salud. Obtencion, caracterizacion, efecto fisiologico y aplicacion en alimentos*; Lajolo, F., Saura-Calixto, F., Wittig de Penna, E., Menezes, E., Eds.; Proyecto CYTED XI.6; Editorial Varela: São Paulo, Brazil, 2001; pp 311−338.

(16) Quackenbush, F. Extraction and analysis of carotenoids in fresh plant materials. *J. Assoc. Off. Anal. Chem.* **1974**, *57*, 511−512.

(17) Granado, F.; Olmedilla, B.; Gil-Martinez, E.; Blanco, I. A fast, reliable and low-cost saponification protocol for analysis of carotenoids in vegetables. *J. Food Compos. Anal.* **2001**, *14*, 479−489.

(18) de Ancos, B.; Gonzalez, E.; Cano, M. P. Effect of high-pressure treatment on the carotenoid composition and the radical scavenging activity of persimmon fruit purees. *J. Agric. Food Chem.* **2000**, *48*, 3542−3548.

(19) Spiller, G.; Chernof, M.; Hill, R.; Gates, J.; Nassar, J.; Shipley, E. Effect of purified cellulose, pectin, and a low-residue diet on fecal volatile fatty acids, transit time, and fecal weight in humans. *Am. J. Clin. Nutr.* **1980**, *33*, 754−759.

(20) Saura-Calixto F.; Goñi, I. The intake of dietary indigestible fraction in the Spanish diet shows the limitations of dietary fibre data for nutritional studies. *Eur. J. Clin. Nutr.* **2004**, *58*, 1078−1082.

(21) Khachik, F.; Goli, M.; Beecher, G.; Holden, J.; Lusby, W.; Tenorio, M.; Barrera, M. Effect of food preparation on quality and quantitative distribution of major carotenoids constituents of tomatoes and several green vegetables. *J. Agric. Food Chem.* **1992**, 40, 390−398.

(22) Prosky, L. What is dietary fiber? *JAOAC Int.* **2000**, *83*, 985−987.

(23) Bravo, L.; Abia, R.; Goñi, I.; Saura-Calixto, F. Possible common properties of dietary fibre constituents and polyphenols. *Eur. J. Clin. Nutr.* **1995**, *49* (Suppl. 3), S211−S214.

(24) Riedl, J.; Linseisen, J.; Hoffmann, J.; Wolfram, G. Some dietary fibers reduce the absorption of carotenoids in women. *J. Nutr.* **1999**, *129*, 2170−2176.

(25) Robert, B.; Horton, P.; Pascal, A.; Ruban, A. Insight into the molecular dynamics of plant light-harvesting proteins *in vivo*. *Trends Plant Sci.* **2004**, *9*, 385−390.

(26) Campbell, J.; Fahey, G. Psyllium and methylcellulose fermentation properties in relation to soluble and insoluble fiber standards. *Nutr. Res.* **1997**, *17*, 619−629.

(27) Cheng, H.; Lai, M. H. Fermentation of resistant rice starch produces propionate reducing serum and hepatic cholesterol in rats. *J. Nutr.* **2000**, *130*, 1991−1995.

Received for review November 26, 2004. Revised manuscript received February 16, 2005. Accepted February 20, 2005. This research was performed with the financial support of the Spanish Comisión Interministerial de Ciencia y Tecnología (Project AGL2002-04104-C04-01), and J.S. thanks the Agencia Española de Cooperación Internacional, Becas MAE, for a scholarship.

JF0480142