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Bioaccessibility of β-Carotene, Lutein, and Lycopene from Fruits and Vegetables

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Epidemiological studies have consistently demonstrated that there is an association between carotenoid-rich food intakes with a low incidence in chronic diseases. Nevertheless, there is not an association between the intake of total dietary carotenoids and chronic health incidence in the European population, probably because of different carotenoid food sources and bioavailability. The objective of this study was to evaluate the small and large intestine bioaccessibilities of major dietary carotenoids from fruits and vegetables in a common diet. A bioaccessibility model that includes enzymatic digestion and in vitro colonic fermentation was employed. Lutein presented greater small intestine bioaccessibility (79%) than β-carotene (27%) or lycopene (40%). With regard to large intestine bioaccessibility, similar amounts of lycopene and β-carotene were released from the food matrix (57%), whereas small amounts of lutein (17%) were released. These results suggest that 91% of the β-carotene, lutein, and lycopene contained in fruits and vegetables is available in the gut during the entire digestion process. Colonic fermentation is shown to be important for carotenoid availability in the gut.

KEYWORDS: β-Carotene; lycopene; lutein; colonic fermentation; bioaccessibility; fruits and vegetables

INTRODUCTION

Epidemiological studies have consistently shown that the consumption of carotenoid-rich foods is associated with a reduced risk of developing several chronic diseases, especially cardiovascular diseases (*1*, *2*) and cancer (*3*−*6*). Carotenoids, long recognized for their antioxidant properties, are of increasing interest because of their effects on regulation of cell growth, modulation of gene expression, and possibly immune response (*7*).

The carotenoid intake in the Spanish Mediterranean-type diet has been reported elsewhere (*8*, *9*). The mean intake of carotenoids in adult populations of Spain (Asturias, Navarra, Guipuzcoa, Murcia, Granada, and Madrid) ranges from 5.88 to 9.54 mg/day (*8*, *9*); the principal food items contributing to this intake are spinach, carrots, lettuces, tomatoes, oranges, and tangerines. Nevertheless, the reported carotenoid intake is much lower in Spain than in other European countries such as the United Kingdom (14.38 mg/day), Ireland (14.53 mg/day), and The Netherlands (13.71 mg/day), where the incidence of chronic diseases is higher (*8*). This lack of association suggests that total carotenoid intake may not be an accurate indicator of carotenoid health effects. The European Prospective Investigation of Cancer (EPIC) study identified several differences between countries with regard to carotenoid food sources with the consequent difference in carotenoid bioavailability. Carotenoid bioavailability may be more closely connected than intake with chronic disease incidence.

Many factors have been found to affect carotenoid absorption after ingestion (e.g., food matrix, dietary fat and fiber content, host-related factors, and interactions with other food constituents) (*10*). The capacity of the digestive process to release carotenoids from the food matrix (namely, bioaccessibility) may be the first step to determine the bioavailability of carotenoids. In this way, the use of in vitro digestion and colonic fermentation models may help provide more insight into the bioavailability of carotenoids from the food matrix and the factors that determine their availability. Little is known about the bioaccessibility of carotenoids from whole foods or whole diets. Moreover, no studies have considered the possibility that carotenoids may also be bioaccessible in the large intestine. In this context, previous results suggest that some carotenoids are available in the small and large intestines, whereas an appreciable amount is unavailable in the gastrointestinal tract (*11*). Enzymatic activity from intestinal bacteria may be involved in the release of bioactive compounds such as carotenoids from the food matrix.

The aim of this work was to estimate the bioaccessibility of major dietary carotenoids from fruits and vegetables included in a common diet in both the small and large intestines, using an in vitro gastrointestinal model that includes a digestive

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Table 1. Carotenoid-rich Fruits and Vegetables Taken as Samples and Their Consumption (Person/Day) in the Spanish Diet

		g of fresh matter[a]	g of edible portion
vegetables	tomato (41.64 g), green bean[b] (7.40 g), cucumber (5.21 g), lettuce (13.27 g), asparagus (1.10 g), spinach[b] (2.82 g), chard[b] (2.44 g), carrot (6.58 g), celery (6.58 g), beet root (6.58 g), green pepper (5.73 g), red pepper (5.73 g), artichoke (6.58 g)	142.56	111.66
fruits	orange (46.63 g), mandarin orange (11.66 g), black olive (3.51 g), green olive (3.51 g), mango (4.05 g), grapefruit (4.05 g)	100.07	73.41

[a] Confidence level 95%, error range 2% in amount of food. [b] Boiled.

enzyme treatment to isolate indigestible food compounds and in vitro colonic fermentation of indigestible food compounds.

MATERIALS AND METHODS

Samples. The most commonly consumed carotenoid-rich fruits and vegetables in the Spanish diet were selected on the basis of dietary sources of carotenoids and food intake according to the literature (*8*, *9*, *12*). The selected items represent >80% of the total intake of carotenoids in the Spanish diet. The amount of every item was based on the estimated intake of that item in the national consumption data (*13*). Products with low or negligible carotenoid content or incidence in the Spanish diet were excluded. Dietary intakes of each food item in the Spanish diet are shown in **Table 1**. Two lots of each individual item were purchased at different local supermarkets. Selected items were grouped into two duplicate samples, vegetable and fruit groups. The edible portion of the daily amount consumed per capita for each item as eaten was weighed: vegetables (total = 111.7 g) and fruits (total = 73.4 g). Each duplicate sample was freeze-dried, ground (particle size = 0.5 mm), and stored at −18 °C until analysis.

Bioaccessibility Model. There are two main steps in the methodology employed to estimate the bioaccessibility of dietary carotenoids (**Figure 1**): (a) isolation of the indigestible fraction (small intestine bioaccessibility) and (b) colonic fermentation of the indigestible fraction (large intestine bioaccessibility). The indigestible fraction was previously defined as the part of plant foods that is not digested or absorbed in the small intestine and reaches the colon, where it serves as a substrate for fermentative microflora (*14*). The indigestible fraction is made up of dietary fiber and other compounds of proven resistance to the actions of enzymes such as indigestible protein, resistant starch, and lignin. It is a physiological alternative to the common dietary fiber concept (*15*). Analytical conditions for indigestible fraction determination are close to physiological conditions (pH, temperature, and incubation times). The indigestible fraction is composed by two fractions: a soluble fraction (supernatant of enzymatic digestion) and an insoluble fraction (residue of enzymatic digestion).

In the in vitro colonic fermentation model, the total indigestible fraction (soluble + insoluble) was fermented in strict anaerobic conditions using rat cecal content as inoculum. Several compounds were released from the food matrix by the action of bacterial enzymes, whereas other compounds remained in the food matrix as a part of the residue after fermentation. The residue after fermentation contains compounds of proven resistance to enzymatic and colonic bacterial degradation, which probably would be excreted in the feces; these comprise the inaccessible compounds.

Indigestible Fraction. The procedure to determine and isolate the indigestible fraction was described by Saura-Calixto et al. (*14*). Twelve samples of each food group (n_1-n_{12}) were successively incubated with digestive enzymes to simulate digestion in the small intestine. Briefly, 300 mg of sample was incubated with pepsin (EC 3.4.23.1, 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 (EC 3.1.1.3, 2 mL of a 7 mg/mL solution in 0.1 M phosphate buffer, pH 7.5, 37 °C, 6 h, Sigma L-3126), bile extract porcine (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 (EC 3.2.1.1, 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). Then samples were centrifuged (15 min, 25 °C, 3000*g*) and supernatants collected. Residues were washed twice with 5 mL of distilled water and all supernatants combined. Residues were stored at −18 °C for colonic fermentation (n_1-n_6) and carotenoids analysis (n_7-n_{12}) (carotenoids associated with the insoluble indigestible fraction). Each supernatant was incubated with 100 μL of amyloglucosidase (EC 3.2.1.3, Roche, 102 857) for 45 min at 60 °C, 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). Retentants contains soluble dietary fiber and other associated compounds such as carotenoids. Dialysis retentates (n_7-n_{12}) were stored at −18 °C for the analysis of carotenoids associated with the soluble indigestible fraction, and the other dialysis retentates (n_1-n_6) were concentrated to 5 mL in an R-114 Büchy vacuum rotatory evaporator and then added to their corresponding residue (insoluble indigestible fraction, n_1-n_6, e.g., soluble indigestible fraction n_1 added to insoluble indigestible fraction n_1) and stored at −18 °C for colonic fermentation.

In Vitro Colonic Fermentation. The in vitro fermentation method was described by Barry et al. (*16*) and standardized by Goñi and Martín-Carrón (*17*). Male Wistar rats (body weight of 200 ± 5 g) fed with standard maintenance diets adjusted to rat nutritional requirements (AO4, Panlab, Barcelona, Spain) were supplied by the Breeding Center at the Faculty of Pharmacy (University Complutense of Madrid, Spain). Rats were killed in a carbon dioxide chamber, and fresh rat cecal contents were used as inoculum. Ceca were removed through abdominal midline incisions. Rat cecal contents were scraped, weighed, and added to a flask containing sterile anaerobic medium to give a 100 g/L inoculum. The anaerobic medium adapted from the method of Goering and Van Soest (*18*) contained trypticase, micromineral and macromineral solutions, and resazurin as anaerobic redox indicator. The inoculum was mixed (10 min) in a Stomacher 80 Lab Blender (Seward Medical, London, U.K.) and filtered (1 mm mesh) before use. Total indigestible fractions from enzymatic treatments (n_1-n_6) were mixed with fermentation medium (8 mL, 4 °C, 16 h). Tubes were sealed with rubber caps (no. 407-0-13, Ormacisa, Madrid, Spain). Two milliliters of inoculum was added and the headspace flushed with carbon dioxide (1 min). Tubes were placed in a shaking water bath (37 °C, 24 h). Blanks containing no substrate and lactulose (Sigma L-7877) were included in the experiment as zero and completely fermentable substrate, respectively. All of the steps were carried out in an oxygen-free CO_2-saturated atmosphere. After incubation time, pH was measured and 1 M NaOH was used to stop the fermentation process. Samples were centrifuged (2500*g*, 10 min, 25 °C), and the supernatants and residue were collected and stored at −80 °C for carotenoids analysis (residue, carotenoids associated with the residue after fermentation; supernatants, carotenoids available after colonic fermentation). The carotenoid contents of supernatants and residue were corrected with blanks of fermentation.

Carotenoid Analysis. Carotenoids were extracted following the procedure described by Quackenbush (*19*) 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*-butyl-hydroxytoluene (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 vortexed vigorously for 30 s and centrifuged (4200*g*, 3 min), and 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. (*20*) with some modifications introduced in our

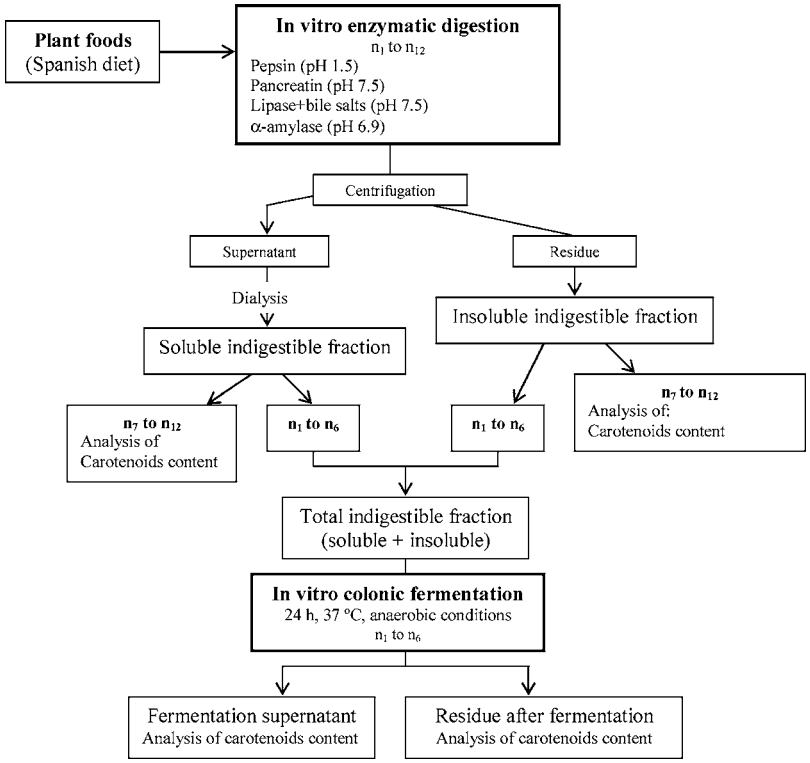

Figure 1. Schematic of the methodology employed to estimate the bioaccessibility of dietary carotenoids. n = number of samples for each food group.

laboratory. Briefly, a mixture containing the carotenoid 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 the ether was colorless (absorbance ≤ 0.001). 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) 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% A and 0% 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), β-carotene (≥95%, Sigma C-4582), and lycopene (≥90−95%, Sigma L9879) were constructed by plotting HPLC peak absorbance area versus concentration of the carotenoid in the injected sample (*21*).

Estimation of β-Carotene, Lutein, and Lycopene Bioaccessibility. To determine the carotenoids' bioaccessibility, the following calculations were used:

Carotenoids bioaccessible in the small intestine were determined as the difference between carotenoid content in the original sample and carotenoids associated with the indigestible fraction.

Carotenoids bioaccessible in the large intestine were determined as the difference between carotenoids associated with the indigestible fraction and carotenoid content in the residue after fermentation.

Statistical Analysis of Data. All data were reported as mean ± standard deviation for at least four replicates in each treatment.

RESULTS AND DISCUSSION

Carotenoid release from the food matrix is a key step in the bioavailability of carotenoids and is affected by both chemical speciation and food matrix. In this study an in vitro method that simulates the human small and large intestine digestion process was used to estimate the amount of carotenoids that becomes accessible from the most commonly consumed carotenoid-rich fruits and vegetables in the Spanish diet. The method estimates the maximum carotenoid release from the food matrix that could be absorbed by the human mucosa in optimal physiological and dietary conditions.

Lutein, β-carotene, and lycopene were selected for analysis because they account for >80% of the total carotenoid intake in the Spanish diet (*8*).

Lutein, lycopene, and β-carotene contents in the vegetable and fruit groups are shown in **Table 2**. β-Carotene is the major carotenoid in both groups. The vegetable group contained 10 times more lutein, lycopene, and β-carotene per dry original sample than the fruit group. This was probably because carotenoid-rich food items in the vegetable group (tomato, carrot, and green leafy vegetable) (*9*) accounted for >45% of the sample.

Small Intestine Bioaccessibility. Comparison of the carotenoid profile before and after enzymatic digestion provides information about carotenoid bioaccessibility in the small intestine. Fruits and vegetables were digested with enzymes (pepsin, α-amylase, pancreatin, lipase-bile salts, and amyloglucosidase). Digestible and nondigestible components were separated, and all nondigestible components were called the food indigestible fraction (FIF) of the samples. The fruit and vegetable groups yielded high FIF contents (55 and 44 g/100 g, respectively). Insoluble components accounted for >90% of the total FIF of these samples, and that is where most of the indigestible carotenoids were found.

The total content and the constituents of the indigestible fraction may affect the enzymatic release of carotenoids during digestion (*11*, *22*). Dietary carotenoids are part of a protein−carotenoid complex, for example, in green leafy vegetables, or a semicrystalline complex, for example, in carrots and tomatoes,

Table 2. Content and Small Intestine Bioaccessibility of Lutein, Lycopene, and β-Carotene in Fruit and Vegetable Groups (Milligrams per 100 g of Dry Edible Portion)

	fruits	vegetables
content		
original sample		
lutein	24.81 ± 2.70	294.60 ± 6.92
lycopene	32.45 ± 0.58	207.71 ± 9.77
β-carotene	40.07 ± 0.64	377.43 ± 7.44
insoluble indigestible fraction		
lutein	16.27 ± 0.10	51.10 ± 0.96
lycopene	30.43 ± 3.91	116.12 ± 1.04
β-carotene	26.03 ± 1.82	275.30 ± 13.10
soluble indigestible fraction		
lutein	0.29 ± 0.01	ND[a]
lycopene	0.51 ± 0.02	0.12 ± 0.01
β-carotene	0.25 ± 0.01	2.78 ± 0.34
bioaccessibility		
carotenoids available in the small intestine[b]		
lutein	8.25	243.50
lycopene	1.51	91.47
β-carotene	13.79	99.35

[a] Not detected. [b] Carotenoid content in the original sample − carotenoid content in the indigestible fraction.

Table 3. Large Intestine Bioaccessibility of Lutein, Lycopene, and β-Carotene in Fruit and Vegetable Groups (Milligrams per 100 g of Dry Original Sample)

	fruits	vegetables
content		
carotenoid content in the total indigestible fraction		
lutein	16.56	51.10
lycopene	30.94	116.24
β-carotene	26.28	278.08
carotenoid content in the residue after fermentation		
lutein	3.11 ± 0.19	9.82 ± 0.27
lycopene	5.15 ± 0.14	3.79 ± 0.13
β-carotene	6.30 ± 0.06	59.22 ± 0.98
bioaccesibility		
carotenoids released from the food matrix by bacterial enzymatic activity[a]		
lutein	13.45	41.28
lycopene	25.79	112.45
β-carotene	19.98	218.86
carotenoids potentially available for absorption in the colon[b]		
lutein	0.81 ± 0.01	1.80 ± 0.21
lycopene	ND[c]	ND
β-carotene	5.02 ± 0.18	34.96 ± 1.23

[a] Carotenoids in total indigestible fraction − carotenoids in the residue after fermentation. [b] Carotenoids in the supernatants after colonic fermentation. [c] Not detected.

and they need to be transferred to a lipophobic domain (enzymatic digesta) before absorption. Therefore, a considerable proportion of dietary carotenoids may remain in the food matrix. Elsewhere the authors have reported the presence of dietary carotenoids associated with FIF (*11*). A large proportion of the carotenoids contained in the original sample remained in the food matrix after the digestive enzyme treatment. The bulk of the nonreleased carotenoids were associated with the insoluble FIF, and a very small proportion (<1%) was associated with the soluble FIF (**Table 2**). These carotenoids were therefore not available after enzymatic treatment and are probably not susceptible to absorption in the small intestine.

The percentage of carotenoid release was lower in fruits than in vegetables. Bioaccessibility of lutein and β-carotene from fruits was significant in the small intestine, and they were released from the food matrix in similar percentages (33 and 34%, respectively). These values were significantly different in the vegetable group, where lutein presented greater bioaccessibility than β-carotene (83 and 26%, respectively). As for lycopene, the bioaccessibilities of the fruit and vegetable groups differed considerably (4 and 44%, respectively).

In general terms, the small intestine bioaccessibility of carotenoids was relatively high as compared with bioavailability studies reported in the literature (*23*, *24*). Lutein presented greater small intestine bioaccessibility than β-carotene and lycopene. In vivo studies confirm these findings, showing much greater relative bioavailability of lutein than of β-carotene (*25*). Castenmiller et al. (*22*) reported similar findings for the relative bioavailability of lutein and β-carotene from spinach (45 and 5%, respectively), probably because lutein is less lipophilic than β-carotene.

The bioaccessible carotenoids in the small intestine are potentially susceptible to absorption 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 interactions with bile acids and dietary fat play a major role in carotenoid bioavailability (*26*). Moreover, the soluble indigestible fraction may interfere with bile salt micelle formation, thus reducing absorption of carotenoids from the food matrix (*27*).

Large Intestine Bioaccessibility. As mentioned above, no studies have addressed the possibility that carotenoids may also be bioaccessible in the large intestine. Carotenoid digestion and absorption in the small intestine are not complete, as many bioavailability studies have shown (*28*, *29*). Therefore, most nondigestible compounds enter the large intestine and provide substrates for the colonic microflora. The metabolic activities in the colon are similar in range to those of the liver. There is a tremendous range in the activity of any enzyme with a particular species, and a wide range of compounds may be released from the food matrix, thus becoming available in the colonic lumen. The bioactive compounds released from the food matrix may interact with the intestinal medium and colonocytes. In that way, as mentioned in a previous publication (*11*), some of the carotenoids released from the food matrix are not utilized as a fermentation substrate by colonic microflora and could be available for absorption in the large intestine (**Table 3**). In this study, 10% of the β-carotene contained in the vegetable group may be available for absorption in the colon. This is a considerable amount if we consider that only 26% of β-carotene in the vegetable group is available after digestive enzyme treatment. With regard to lutein availability in the large intestine, only small amounts were available after the fermentation process, and no lycopene was detected. At this time there are no data available on carotenoid absorption in the large intestine. Gireesh et al. (*30*) found that colonic epithelial cells contained appreciable amounts of β-carotene, but the origin of the β-carotene (blood stream or dietary) is unknown.

To estimate carotenoid bioaccessibility in the large intestine, the residue after fermentation was analyzed for carotenoid content. The residue after fermentation contains compounds of proven resistance to enzymatic and colonic bacterial degradation, which are probably excreted in the feces and comprise the unavailable compounds. The amount of carotenoids associated with the residue after fermentation is shown in **Table 3**. The percentages of lutein, lycopene, and β-carotene not used as fermentative substrates in fruits (1.4, 2.7, and 2.1%, respectively) were similar. Moreover, the residues after fermentation of the

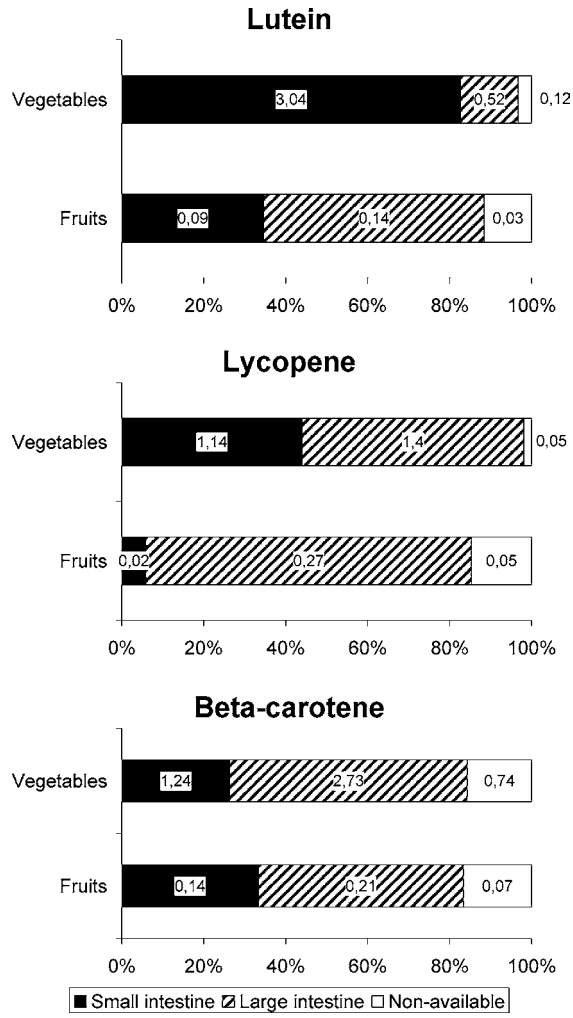

Figure 2. Small and large intestine bioaccessibility of lutein, lycopene, and β-carotene in fruit and vegetable groups. Values within bars indicate the estimated intake of carototenoids (mg/person/day) from fruits and vegetables in the Spanish diet.

vegetable group contained more β-carotene (27%) than lycopene (14%) or lutein (5%).

Carotenoid bioaccessibility in the large intestine is shown in **Table 3**. Note the amount of carotenoids released from the food matrix during colonic fermentation. For example, in the fruit group >70% of lycopene was released during colonic fermentation. The results were similar for β-carotene, for which at least 50% was released by colonic bacteria metabolism in both groups. On the other hand, lutein bioaccessibility in the large intestine was low (14% in the vegetable group). In a way, our results suggest associations similar to those observed by Slaterry et al. (*31*) regarding lutein intakes and low incidences in proximal colon cancer. The high small intestine bioaccessibility of lutein combined with its low absorption would yield large amounts of lutein in the proximal colon. On the other hand, the association of high intakes of β-carotene with a low incidence of distal colon cancer (*31*) would probably be due to high release of β-carotene from the food matrix during colonic fermentation. Such high large intestine bioaccessibility of β-carotene may help to counteract the oxidizing compounds generated during colonic fermentation, especially in the distal colon.

Concluding Remarks. The results of this coupled system demonstrate that the release of carotenoids from the food matrix of vegetables and fruits is sequential, produced first by digestive enzymes and later by enzymatic activity from intestinal microbiota. Therefore, some carotenoids may be absorbed in the small intestine epithelium, some may become available through the colonic epithelium, and the rest would be excreted in the feces.

Figure 2 summarizes the estimated percentages of β-carotene, lutein, and lycopene that are available in the gastrointestinal tract, along with the percentage of small and large intestine bioaccessibility. Our findings suggest that lutein is more bioaccessible in the small intestine; β-carotene is released from the food matrix largely in the large intestine, wheres lycopene presents similar food matrix releases in both compartments. The large intestine may be an important site of carotenoid availability in the gut. Further research is necessary to elucidate the magnitude and the mechanics of carotenoid absorption in the large intestine.

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