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Comparative in Vitro Bioaccessibility of Carotenoids from Relevant Contributors to Carotenoid Intake

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To compare the in vitro bioaccessibility of lutein, zeaxanthin, β-cryptoxanthin, lycopene, and α-and β-carotenes from relevant dietary contributors, a gastrointestinal model was used to assess the stability, isomerization, carotenol ester hydrolysis, and micellarization. Salivar, gastric, duodenal, and micellar phases were extracted, with and without saponification, and analyzed by using a quality-controlled HPLC method. The stability of carotenoids under digestion conditions was >75%, regardless of the food analyzed, whereas micellarization ranged from 5 to 100%, depending on the carotenoid and the food. *cis*-Isomers were maintained in processed foods, but increased in fresh foods. Xanthophyll ester hydrolysis was incomplete (<40%), and both free and ester forms were incorporated into supernatants, regardless of the xanthophyll involved and the food assessed. In vitro bioaccesibility varies widely both for different carotenoids in a given food and for a given carotenoid in different foods. Although in vitro bioaccesibility may not be enough to predict the in vivo bioavailability, it may be relevant for the food industry and for food-based dietary guidelines.

KEYWORDS: Bioaccessibility; carotenoids; xanthophyll esters; in vitro digestion

INTRODUCTION

Fruits and vegetables are the major sources of biologically active compounds (i.e., phytochemicals), and an increased consumption is recommended. Among these compounds, the carotenoids constitute an important group in human diets and display, in addition to their vitamin activity, several other biological activities including antioxidant capacity, blue light filtering, modulation of immune function, and regulation of cell differentiation and proliferation (*1*−*3*).

In most populations, fruits and vegetables provide about 70−90% of the carotenoids in the diet in both developed and developing countries (*4*), and the consumption of a few fruits and vegetables accounts for most of the variability in the carotenoid intake within a population (*5*−*7*). In addition, the food industry is playing an increasing role in the development and marketing of new products with added nutritional value, even though their potential impact on public health and the nutritional status of the population are uncertain.

Bioavailability is a critical feature in the assessment of the role of dietary components in human health. Interest in the bioavailability of vitamins and other phytochemicals has greatly increased because of the existence of undernourished populations and groups at risk of developing micronutrient deficiencies (i.e., the elderly) and the epidemiological evidence suggesting protective effects against noncommunicable diseases (i.e., cancer, cardiovascular disease, age-related eye diseases) (*8*).

The study of the bioavailability of food components can be addressed through a broad approach involving in vitro and in vivo methods, each having their pros and cons. In vitro models to assess carotenoid bioaccessibility and bioavailability (including models of cellular uptake) are increasingly being used to study pre-absorptive processes and food-related factors affecting carotenoid bioavailability (*9*−*11*). Within this context, our aim was to compare the in vitro bioaccessibility of carotenoids from foods contributing substantially to their intake in the Spanish population, considering different food-related factors known to be relevant determinants of the carotenoid bioavailability in foods (*12*), that is, food matrix, type and amount present, and chemical forms.

MATERIALS AND METHODS

Carotenoid-Rich Fruits and Vegetables. Selection of the food items was based on (1) past and current data regarding the contribution of individual fruits and vegetables to the carotenoid intake in Spain (*7*, *13*), including some items known to be good sources (i.e., loquat, corn); and (2) the consideration of food-related factors affecting the bioavailability of carotenoids from foods (*12*), such as the food matrix, the type and amount of carotenoids, and the major chemical forms present

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Table 1. Fruits and Vegetables Studied According to the Food Matrix and the Major Chemical Forms Present

	food matrix		
major chemical form	green vegetables	non-green vegetables	fruits
free carotenoids[a]			
lutein	spinach, lettuce, broccoli	sweet corn, carrot, tomato puree	kiwi
zeaxanthin	spinach, lettuce	sweet corn	
α-cryptoxanthin		sweet corn	
β-cryptoxanthin			
lycopene		tomato puree	
α-carotene		carrot	
β-carotene	broccoli, spinach, lettuce	tomato puree, carrot, red pepper	kiwi, loquat, pineapple
carotenol esters			
lutein esters		red pepper	orange
zeaxanthin esters		red pepper	orange
α-cryptoxanthin esters		red pepper	
β-cryptoxanthin esters		red pepper	orange, loquat, pineapple

[a] Contain <10% as ester forms. Lettuce (*Lactuca sativa*) (fresh); spinach (*Spinacea oleracea*) (frozen, microwaved); broccoli (*Brassica oleracea*) (fresh, microwaved); sweet corn (*Zea mays*) (tinned); carrot (*Daucus carota*) (fresh, boiled); tomato puree (*Lycopersicon esculentum*) (tinned); red pepper (*Capsicum annuum*) (tinned); kiwi (fresh) (*Actinidia chinensis);* orange (*Citrus sinensis*) (fresh); loquat (*Eriobotrya japonica*) (tinned); pineapple (*Ananas comosus*) (fresh).

(free vs ester forms). The selected foods are shown in **Table 1** and, in all, contributed between 65 and 92% to the dietary intake of individual carotenoids from fruits and vegetables in Spain (*7*, *13*).

Standards and Reagents. Unless otherwise stated, all reagents and materials were purchased form Sigma Aldrich Química, VWR Internantional Eurolab, and Carlo Erba (Spain). Zeaxanthin, β-cryptoxanthin, phytoene, and 9-*cis*- and 13-*cis*-β-carotene were generously supplied by DSM (formerly Hoffmann-La Roche, Basel, Switzerland).

In Vitro Digestion. Stability (recovery, isomerization), the degree of ester hydrolysis, and the incorporation into supernatants were assessed using a previously tested in vitro gastrointestinal model (*14*). Briefly, fruits (fresh or tinned) and vegetables (microwaved, boiled, or tinned) were homogenized with a kitchen blender for 1 min to simulate mastication, regardless of the final particle size achieved. Samples (in triplicate) of ca. 10 g were transferred to a flask, and a saliva solution (9 mL), at pH 6.5, containing organic and inorganic components, and α-amylase (145 mg) were added, after which they were incubated in a shaking water bath (37 °C, at 95 opm) for 5 min. Gastric juice (13.5 mL) with organic and inorganic solutions, mucin (1 g), bovine serum albumin (1 g), and pepsin (1 g) from porcine stomach were added. The pH was adjusted to 1.1 and the solution incubated for 1 h. Duodenal juice [25 mL, organic plus inorganic solutions, containing porcine pancreatin (3 g)] and bile solution [containing bovine bile (0.6 g)] were introduced after neutralization of the pH (7.8), and human pancreatic lipase (1 unit), colipase (12.5 μg), cholesterol esterase (5 units), phospholipase A_2 (50 μL), and taurocholate salts (19.9 mg) were added. The final volume was ca. 65 mL, and the mixture was incubated for 2 h.

It was considered that the carotenoid content in the supernatants and residues reflects the amounts of compounds available in the small and large intestine, respectively (*15*, *16*). Thus, transfer from the duodenal digesta to the aqueous-micellar phase was estimated by calculating the proportion of (free) carotenoids in the supernatants. Overnight decantation (at room temperature) was used to produce the supernatants because this approach rendered better estimates than low-speed centrifugation (*14*).

The analysis of carotenoids was performed in aliquots collected from the starting material (ready-to-eat food) and at different time points during the in vitro digestion (salivary, gastric, duodenal phase, and after incorporation into supernatants). At the end of each phase, aliquots (ca. 1 mL) were collected in duplicate, extracted before and after saponification with KOH (40% in methanol, vortex for 5 min), and analyzed by HPLC (*17*, *18*).

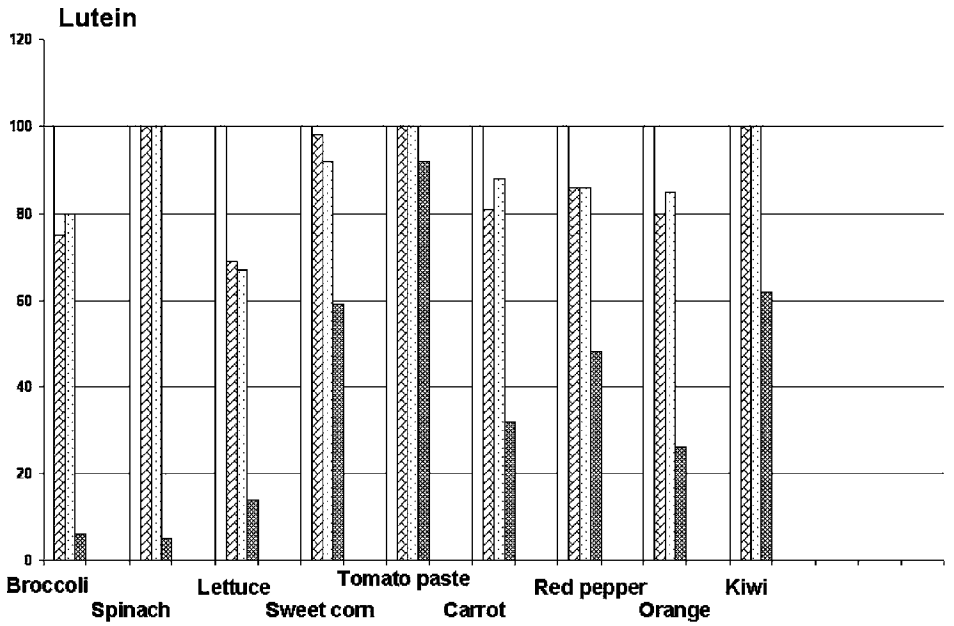
Analytical Conditions. The chromatographic system consisted of a Spheri-5-ODS column (Applied Biosystems, San José, CA) with gradient elution of acetonitrile/methanol (85:15, by vol) for 5 min to acetonitrile/methylene chloride/methanol (70:20:10, by vol) for 20 min. Ammonium acetate (0.025 M) was added to the methanol. Detection was carried out by a photodiode array (model 996, Waters Associates, Milford, MA) set at 450, 370 (for phytofluene), and 286 nm (for phytoene). Using this method, *trans*-lutein, zeaxanthin, 13- or 15-*cis*-lutein, α-cryptoxanthin, β-cryptoxanthin, *trans*-lycopene, 5-, 7-, or 9-*cis*-lycopene, 13-*cis*-lycopene, 15-*cis*-lycopene, γ-carotene, α-carotene, *all-trans*-β-carotene, 9-*cis*-β-carotene, 13- or 15-*cis*-β-carotene, phytofluene, and phytoene can be simultaneously determined. Additional tentatively identified carotenoids that were resolved, if present, included violaxanthin, neoxanthin, and capsanthin. The identification of the compounds was carried out by comparing retention times with those of authentic standards, on-line UV−vis spectra, and chemical reactions (i.e., alkaline hydrolysis, reaction with diluted hydrochloric acid).

Within-day variability of the major compounds (i.e., lutein, β-cryptoxantin, β-carotene, and α- and γ-tocopherols) during the in vitro digestion was, on average, <10%, although the degree of matrix homogenization (i.e., particle size) affected the precision (*14*). The short- and long-term precision and accuracy of the analytical method was within accepted values, as verified periodically through our participation in the Fat-Soluble Quality Assurance Program conducted by the National Institute of Standards and Technology (NIST; Gaithersburg, MD).

Calculations. Carotenoid content is expressed in micrograms per 100 g of edible portion and corresponds to mean values from analyses performed in triplicate. However, to enable the comparability of the results of different experiments and matrices, the parameters evaluated (i.e., stability, extent of hydrolysis, isomerization, transfer to supernatants) are expressed as percentages of the initial total (saponified) content of carotenoids in the food. The results were interpreted on the basis of data from crude (i.e., extent of hydrolysis) and saponified extracts (i.e., stability). Using each food as a single data point, Pearson and Spearman correlation coefficients were calculated to assess relationships between the amounts of carotenoids transferred to the supernatants and the fat and fiber content of the foods, as referenced in *Food Composition Tables* (*19*, *20*).

RESULTS

Stability and Isomerization. Stability was calculated in saponified extracts and, thus, the values correspond to the sum of free and ester forms remaining in a given phase, compared to the initial total content. As shown in **Figure 1**, stability during salivary, gastric, and duodenal phases was, on average, >80% for all of the carotenoids evaluated (except in lettuce), regardless of the type of food matrix (green or non-green vegetable or fruit). Also, with the exception of lettuce and fruits (fresh foods), the cis/trans ratio in processed foods was apparently unaffected during the gastric and duodenal phases (**Figure 2**).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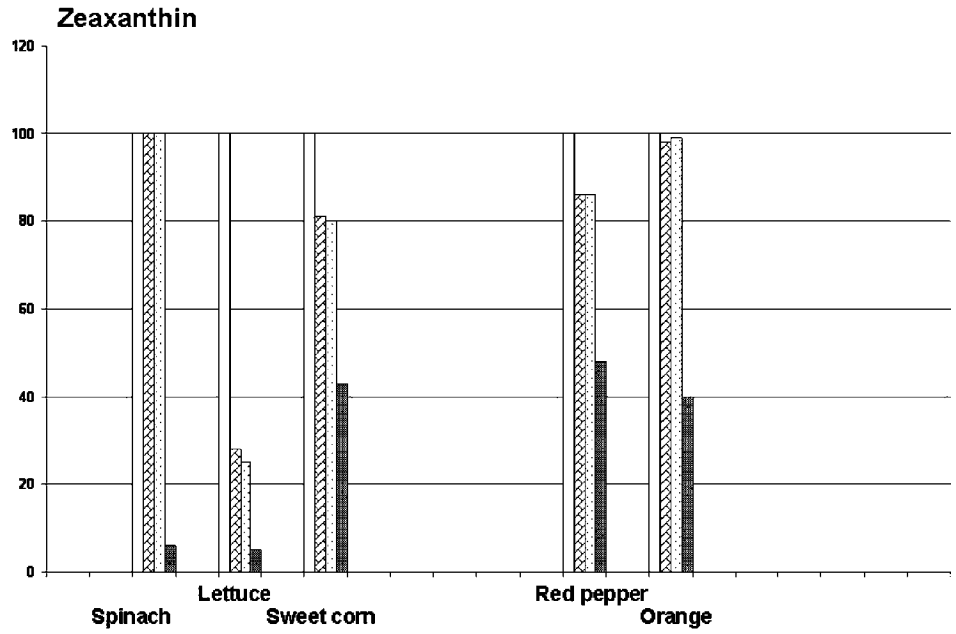
Broccoli
Spinach
Lettuce
Sweet corn
Tomato paste
Carrot
Red pepper
Orange
Kiwi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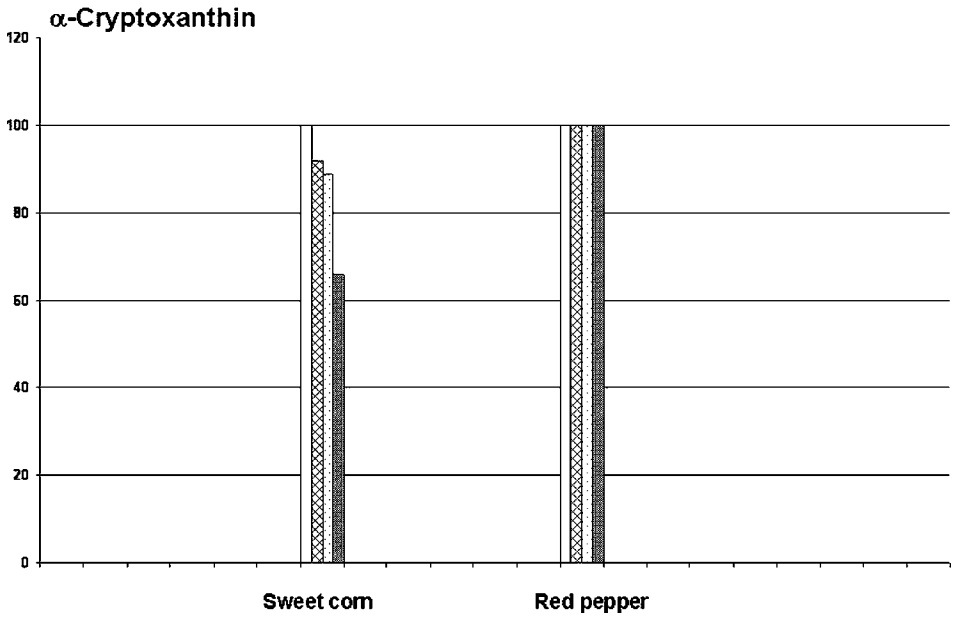
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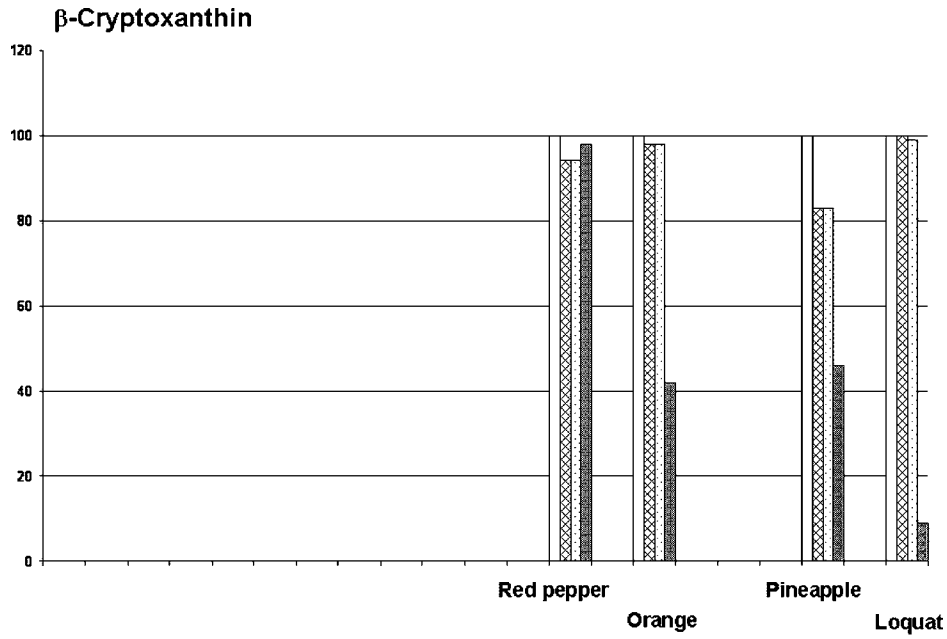
Sweet corn
Red pepper


β-Cryptoxanthin
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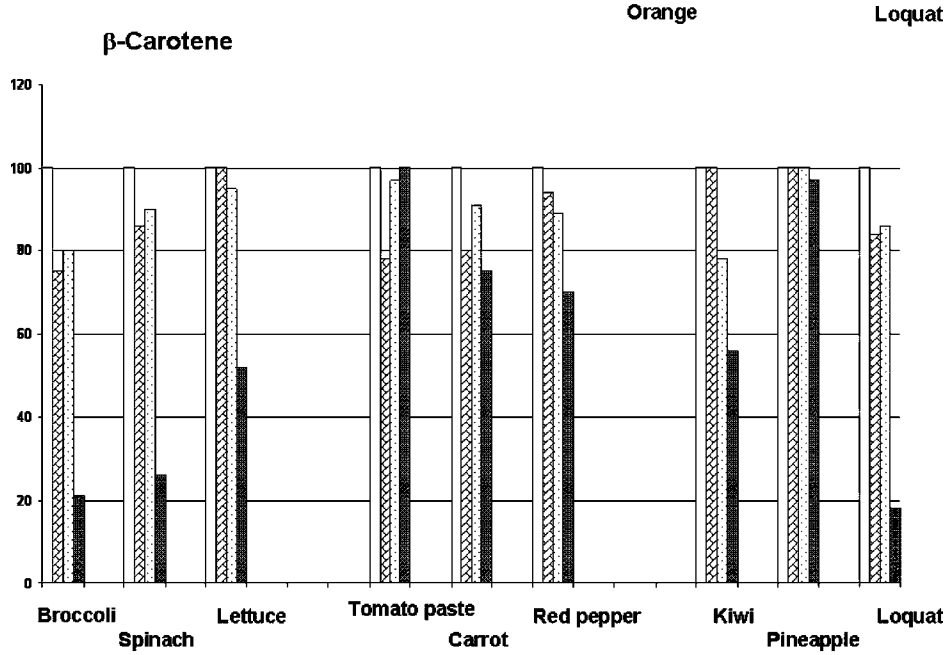
Pineapple
Loquat


β-Carotene
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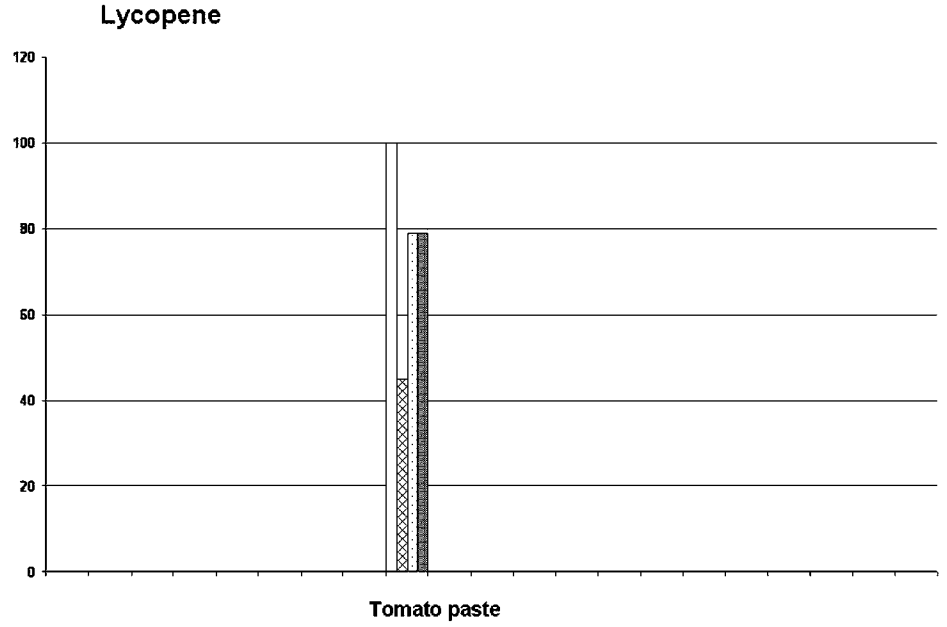
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Tomato paste

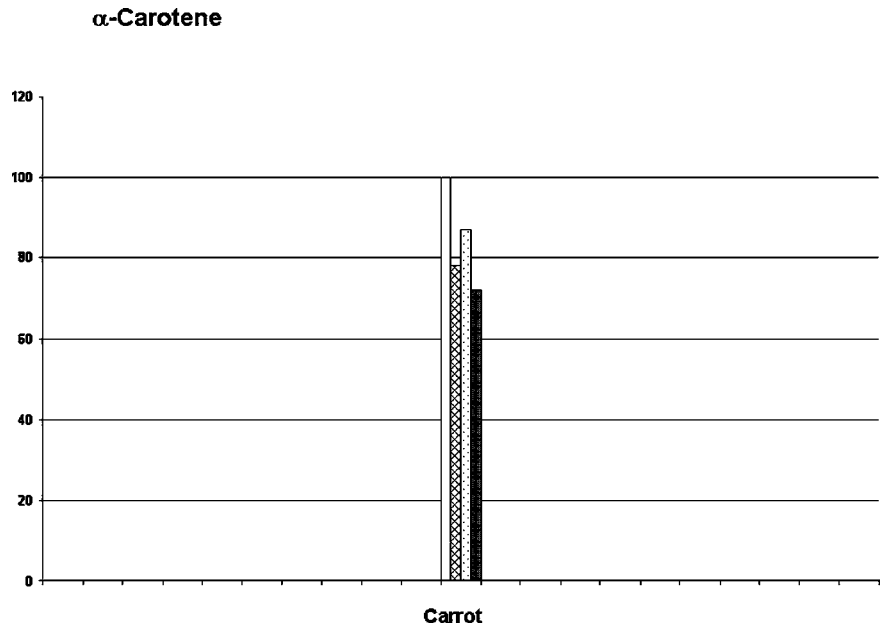

Figure 1. Stability (percent) and accessibility (percent, micellar phase) during in vitro digestion: food (white bars), gastric phase (brick-patterned bars), duodenal phase (dotted bars), and transferred into supernatants (cross-hatched bars). All percentages refer to the proportion of the total initial content in the food (saponified extracts).

Table 2. Carotenoid Content (Micrograms per 100 g of Edible Portion) in the Foods and Recovered at the Micellar Phase (Accessibility) after in Vitro Digestion[a]

	lutein (trans)		zeaxanthin		α-cryptoxanthin		β-cryptoxanthin (trans)		lycopene (total)		α-carotene		β-carotene (total)	
	food	micellar	food	micellar	food	micellar	food	micellar	food	micellar	food	micellar	food	micellar
green vegetables														
broccoli	1305	78											794	134
spinach	7142	334	119	8									662	172
lettuce	248	34	53	3									96	50
non-green vegetables														
sweet corn	120	71	150	81	50	33								
fresh tomato	83	76							6441	5113			215	214
carrot	354	117									1294	932	3230	2434
red pepper	533	353	430	208	52	62	314	307					618	434
fruits														
orange	31	8	18	7	46	19								
kiwi	95	59											31	17
pineapple							6	3					26	25
loquat							469	41					222	38

[a] Carotenoid content in the food refers to saponified extracts. Amounts quantified in the micellar phase correspond to free forms incorporated.

Carotenol Ester Hydrolysis. Carotenol esters were hydrolyzed during the duodenal phase. The amounts of free xanthophylls initially present varied widely according to the food and the xanthophyll concerned, ranging from <10% in loquat (β-cryptoxanthin) and 18−26% in orange (for lutein, zeaxanthin, and β-cryptoxanthin) to 30, 47, 56, and 84% in red pepper (for lutein, zeaxanthin, β-cryptoxanthin, and α-cryptoxanthin, respectively). Under the conditions employed, hydrolysis was incomplete, even though the amounts of the free forms in the duodenal phase could represent up to 16-fold of those initially present in the food. Also, the extent of "net" hydrolysis (after correction for initial content) was highly variable both for different xanthophylls in a given food (i.e., 3% for zeaxanthin and 30% for lutein in red pepper) and for a given xanthophyll in different foods (<1% of β-cryptoxanthin in red pepper and >90% in pineapple). Although the differential hydrolysis of monoester and diester forms, if present, was not assessed, the chromatographic profile after the duodenal phase was qualitatively the same as that initially present in the food, but with lower amounts of ester forms and greater amounts of the corresponding free xanthophylls, including those tentatively identified but not quantified (i.e., neoxanthin, violaxanthin, capsanthin). Finally, it should be noted that free forms in the duodenal phase actually provide a composite of both hydrolysis and stability and, therefore, the "true" extent of hydrolysis may be higher.

Transfer to Supernatants. The extent of incorporation into supernatants varied from one carotenoid to another in a given food, as well as for each carotenoid in the different foods. On a group basis, non-green vegetables showed overall higher rates of transfer of both carotenes and xanthophylls than fruits, with green vegetables showing the lowest rate of all, both for lutein (and zeaxanthin) and for β-carotene (**Figure 1**; **Table 2**). In foods containing carotenol esters, both free and ester forms of different xanthophylls (i.e., lutein, zeaxanthin, β-cryptoxanthin, violaxanthin, capsanthin) were consistently present in the

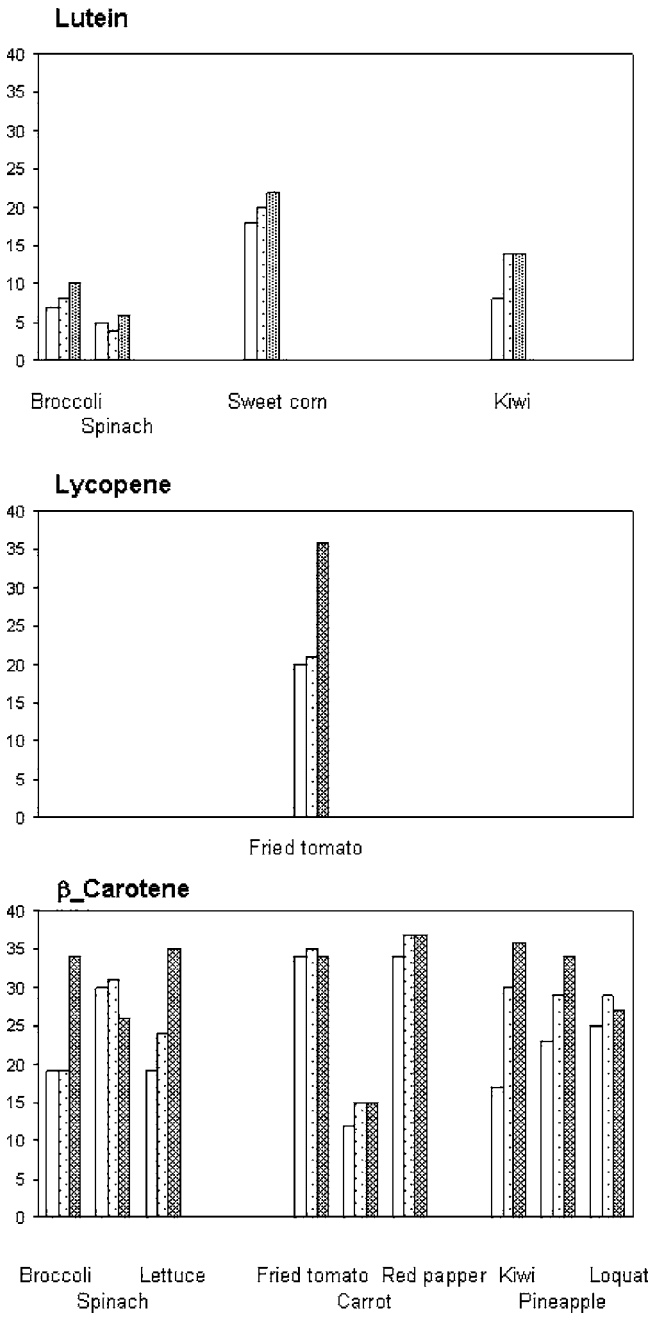

Figure 2. Percentual contribution of *cis*-isomers to the total content initially present in the foods (white bars), at the end of the duodenal phase (dotted bars), and transferred into supernatants (cross-hatched bars).

supernatants. *cis*-Isomers were also transferred to the supernatants and, although a higher transfer efficiency of *cis*-forms was apparent in some foods (i.e., β-carotene in broccoli and kiwi, *cis*-lycopene in tomato puree), a consistent preferential pattern of incorporation of *cis*- versus *trans*-isomers was not observed (**Figure 2**).

Finally, although not significant, negative correlations were observed between fiber content and the transfer of lutein, β-carotene, and β-cryptoxanthin, and positive correlations were found between fat content of the food and the transfer of β-cryptoxanthin, β-carotene, and, especially, lutein (Spearman $r = 0.59$, $p = 0.09$, using fat content from ref *19*; $r = 0.70$, $p = 0.08$, using fat data from ref *20*). Considering all of the foods analyzed, only lutein and β-carotene were correlated during transfer to supernatants ($r = 0.86$, $p = 0.014$).

DISCUSSION

Carotenoid bioavailability is known to be greatly affected by several food-related factors (*12*). In green leafy vegetables, carotenoids are associated with the light-harvesting complex in the thylakoid membranes of the chloroplasts, whereas in roots and fruits, carotenoids occur as membrane-bound semicrystalline structures. Such a complex environment has major implications for their extraction, analysis, and behavior during digestion (*21*).

Under the gastrointestinal conditions employed, we found that carotenoids showed a relatively high stability with, on average, >80% remaining in the final digesta, as previously reported (*9*, *10*, *16*, *22*). In the present study, the cis/trans ratio was relatively high in tinned and cooked foods, probably because both processes involve thermal processing. However, the cis/trans ratio in these items was apparently unaffected during the gastric and duodenal phases, coinciding with findings observed in vitro (*23*) and in cannulated humans (*24*). In contrast, an increase in the cis/trans ratio during the in vitro digestion (including the transfer to supernatants) was observed in fresh items (i.e., kiwi, pineapple, lettuce), being more apparent in foods containing free carotenoids (i.e., lettuce, kiwi) than in those having ester forms (i.e., pineapple, loquat). This fact may be related both to a higher extractability/solubility (free carotenoids) and to the stability under the conditions employed (i.e., greater for ester forms), although the preferential micellarization of *cis*-isomers has been already observed (*23*, *25*).

Regardless of the food studied, hydrolysis of carotenol esters was incomplete, with average estimates of ca. <40%, as previously observed (*14*, *25*). Moreover, in the present study, although different xanthophyll monoesters and diesters were cleaved, the extent of "net" hydrolysis was highly variable for different xanthophylls in a given food, as well as for a given xanthophyll in different foods. This suggests that cholesterol esterase lacks specificity to effectively hydrolyze different xanthophyll esters, as previously observed (*26*), although, because the amount of free xanthophylls in the final digesta should be interpreted as a composite of hydrolysis and stability, some degree of cleavage specificity and/or preferential stability cannot be ruled out.

Whereas the stabilities of carotenoids were, on average, comparable among the foods evaluated, the degree of incorporation into supernatants (micellarization), showed striking differences. Compared to non-green vegetables and fruits, green vegetables showed the lowest rate of transfer of all the carotenoids (i.e., lutein, zeaxanthin, and β-carotene), a finding consistent with observations in human studies (*27*). Nevertheless, we used overnight decantation to produce the supernatants, and it could be that digestion actually continued rendering greater amounts of free forms available for transfer. However, this is unlikely because, under the conditions employed, the extent of hydrolysis did not increase when the incubation time was lengthened (*14*) and overnight decantation was performed at room temperature, far from the optimal conditions for enzyme activity.

The lower rate of transfer in green vegetables may be related to the cellular localization and, thus, to the extractability/stability of carotenoids (*21*) and to other components in the media (i.e., lysophosphatidylcholine/phosphatidylcholine ratio) that may modify substantially the transfer efficiency for some carotenoids (i.e., lutein) (*10*, *28*). Also, an effect of absorption modifiers (i.e., fat, fiber) could be expected, and, for example, a significant correlation between the availability of carotenoids (lutein and β-carotene) in the small intestine and the content of Klason lignin, non-starch polysaccharides, and resistant protein has been

reported in green leafy vegetables (*16*). However, in the present study, although the degree of incorporation showed both a positive and a negative trend with respect to the fat and fiber content of the foods, respectively, none of these relationships reached statistical significance or fully explained the different transfer efficiency observed across the different foods analyzed. In addition, due to the experimental protocol used and because carotenoids may be also accessible in the large intestine due to the interaction with colonic microflora (*16*), values in the micellar phase should be interpreted in terms of bioaccesibility in the small intestine and not necessarily as the total bioaccesibility in the whole intestine.

Xanthophylls have been reported to be more efficiently transferred than carotenes (*10*, *22*), although transfer efficiency varies among foods (*11*). Overall, our results support the idea that the efficiency of micellarization and/or the apparent preferential incorporation of xanthophylls may be strongly dependent on the type of matrix (and on the composition of the final digesta) in which they are contained, as previously reported (*11*, *12*, *16*, *24*, *29*). It is also known that carotenoids interact during incorporation and absorption (*30*, *31*) and, thus, the type and the relative amounts of carotenoids present in the final digesta (i.e., free versus ester forms) may become relevant. The transfer of carotenoids may be prevented because they are deeply embedded in the food tissues and because of interfacial characteristics (i.e., surface-active proteins) or electric charges (ζ-potential) (*31*). Thus, the specific chemical composition of the final digesta for each food may provide an enhancing (i.e., incomplete hydrolysis and, thus, the presence of ester forms as an adjuvant lipid vehicle) or an inhibitory (high fiber content) microenvironment for the transfer of carotenoids to micelles. Consistently, it has been reported that the hydrolysis of zeaxanthin esters during the small intestinal phase of (in vitro) digestion enhances zeaxanthin bioavailability (*25*). In this regard, the food-related environment could partly explain the differential transfer efficiency of lutein and β-carotene from different foods (i.e., green vs non-green vegetables), the correlated incorporation of lutein and β-carotene across the foods analyzed, or the high bioaccessibility of carotenoids from tomato puree (thermally processed and with a higher fat content), an observation consistent with the data available in humans (*32*).

Finally, the incorporation of different xanthophyll esters into supernatants was consistently observed in those foods containing ester forms, confirming recent reports (*14*, *25*). In this sense, our data regarding red pepper suggest that ca. 68% of the zeaxanthin and 80% of the lutein present in the supernatants existed as free forms, meaning that ca. 20−30% were incorporated as ester forms. Although these values are higher than the uptake of zeaxanthin monoesters reported for Caco-2 cells (ca. 8%) (*25*), hydrolysis of xanthophyll esters by esterases at the brush border prior to absorption has been also observed (*21*).

In summary, the stability of carotenoids is high regardless of the plant food assessed, but in vitro bioaccesibility varies widely for different carotenoids in a given food as well as for a given carotenoid in different foods. Nowadays, in vitro models are increasingly used, and it has been suggested that the in vitro approach may be suitable for predicting the bioavailability of phytochemicals from foods (*11*, *33*), although exceptions do exist and in vitro data are not fully concordant with in vivo responses (*33*, *34*). Thus, although in vitro assays may be relevant in the food industry and to the development of food-based dietary guidelines, results should be interpreted with caution because in vitro bioaccesibility may not be enough to predict in vivo bioavailability.

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