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ScienceDirect
Food Chemistry 102 (2007) 641–648
Food Chemistry
www.elsevier.com/locate/foodchem

In vitro bioaccessibility of carotenoids and tocopherols from fruits and vegetables

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Received 8 February 2006; received in revised form 4 May 2006; accepted 21 May 2006

Abstract

Aim of the study: To assess the *in vitro* bioaccessibility of carotenoids, including xanthophyll esters, and tocopherols from fruits and vegetables.

Results: Stability for carotenoids and tocopherols was over 70%. Xanthophyll esters were cleaved by cholesterol esterase but not by human pancreatic lipase. Less than 40% of the β-cryptoxanthin initially present was hydrolyzed and the amount of free xanthophylls recovered was higher when liquid was used than when fresh homogenized matrix was employed. *cis*-Isomers of β-carotene and lutein did not significantly increase during the process. Xanthophylls were more efficiently transferred into supernatants than tocopherols and β-carotene. *cis*-Carotenoids, epoxy-xanthophylls and ester forms were also transferred.

Conclusion: The results are consistent with observations in other *in vitro* digestion models and human studies and support the usefulness of *in vitro* assessment to study food-related determinants of the bioavailability of carotenoids and tocopherols from fruits and vegetables.

Keywords: Bioaccessibility; Carotenoids; Tocopherols; *In vitro* digestion; Xanthophyll esters

1. Introduction

Fruits and vegetables are major sources of biologically active compounds (i.e., phytochemicals). Among these, carotenoids and tocopherols constitute important groups in human diets that display several biological activities, including vitamin activity, antioxidant capacity, blue light filtering, modulation of immune function and regulation of cell differentiation and proliferation (Beatty, Boulton, Henson, Koh, & Murray, 1999; Bendich & Olson, 1989; Bertram & Bortkiewicz, 1995; Jiang, Cristen, Shigenaga, & Ames, 2001).

Interest in the bioavailability of vitamins and other food components (i.e., carotenoids) has greatly increased for different reasons, including the existence of undernourished populations worldwide and groups at risk of developing micronutrient deficiencies (i.e., the elderly), the epidemiological evidence suggesting protective effects against several diseases (i.e., cancer, cardiovascular diseases, cataracts, neural tube defects) and the huge emerging role of the food industry in developing new products with added nutritional value, and their potential impact on public health and the nutritional status of the population (Granado-Lorencio & Olmedilla-Alonso, 2003).

Bioavailability is a critical concept in the assessment of the role of vitamins in human health. However, it comprises different steps and, thus, the amount of a food component that is released from the food matrix is commonly referred to as bioaccessibility (“digestibility”) and, for some components, constitutes the maximum amount available for absorption. In addition, fat-soluble components must be incorporated into mixed micelles before

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0308-8146/$ - see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2006.05.043

absorption. Thus, the efficiency of micellization (quantities transferred into the aqueous-micellar fraction) is used as an estimate of the relative bioavailability of carotenoids, whereas, in combination with the utilization of the Caco-2 cell line as a surrogate for small intestinal enterocytes, it has been employed as a model for human absorption (Failla & Chitchumroonchokchai, 2005).

In vitro models based on human physiology have been developed as simple, inexpensive and reproducible tools to study digestive stability, micellization, intestinal transport and metabolism and to predict the bioavailability of different food components (i.e., ascorbic acid, carotenoids, glucosinolates, polyphenols) (Chitchumroonchokchai, Schwartz, & Failla, 2004; During, Hussain, Morel, & Harrison, 2002; Garret, Failla, & Sarama, 1999; Hedren, Mulokozi, & Svanberg, 2002; Serrano, Goñi, & Saura-Calixto, 2005; Sugawara et al., 2001; Tyssandier et al., 2003; Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004). However, several food and host-related factors are capable of influencing vitamin bioavailability at different points (West & Castenmiller, 1998) and, thus, *in vitro* methodology for bioavailability assessment and its potential predictive value regarding human absorption of phytochemicals should be validated in different in vivo situations.

Fruits containing xanthophyll esters (i.e., loquat, oranges) are good sources of provitamin A carotenoids and, in many countries, constitute the best source to cover vitamin A requirements (De Pee et al., 1998; Rodriguez-Amaya, 1997) although, to date, few studies have examined the effect of carotenoid esterification (Pérez-Gálvez & Mínguez-Mosquera, 2005). To our knowledge, with the exception of the use of extracts in enzymatic assays (Breithaupt, Bamedi, & Wirt, 2002), food matrices containing xanthophyll esters have scarcely been evaluated in gastrointestinal models. Interestingly, in humans, the plasma response to a single dose of xanthophyll esters, compared to free forms, has provided both similar (for β-cryptoxanthin) and higher bioavailability measures (for zeaxanthin esters) (Breithaupt, Weller, Wolters, & Hahn, 2003; Breithaupt, Weller, Wolters, & Hahn, 2004). Thus, our aim was to assess simultaneously the bioaccessibility of carotenoids, including xanthophyll esters, and tocopherols from fruits and vegetables and, specifically, to study their stability, isomerization, hydrolysis and transfer into aqueous phase.

2. Methods and materials

2.1. 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, 9-*cis*- and 13-*cis*-β-carotene were generously supplied by DSM (formerly Hoffmann-La Roche, Basel, Switzerland), whereas neoxanthin and violaxanthin were supplied by Dr. Tóth (Pécs, Hungary).

2.2. In vitro digestion model

Since previously reported models were found to be unsuitable for xanthophyll ester hydrolysis (Garret et al., 1999; Hedren et al., 2002), our approach was to modify a complete static gastrointestinal protocol (Oomen et al., 2003) that, although originally applied to the evaluation of soil contaminants, was validated in interlaboratory comparative studies against both static and dynamic *in vitro* models (Oomen et al., 2002).

Compositions and concentrations of inorganic and organic solutions, gastric and duodenal juices and bile constituents were carefully duplicated as described by Oomen et al. (2003). Compared to this and previous *in vitro* models for carotenoids, the modifications included the use of human pancreatic lipase (EC 3.1.1.3) and cholesterol esterase (EC 3.1.1.13), tested independently and combined at different concentrations, phospholipase A_2 (EC 232.637.7) and taurocholate salts. We used human pancreatic lipase since the source of the lipase (i.e., microorganisms, porcine, human) affects the degree of xanthophyll ester hydrolysis (Breithaupt et al., 2002) and, thus, its incorporation may be regarded as more physiological than that of other non-human lipases previously used. Also, cholesteryl esterase (CE) was added because of the inability of previous and present methods (using pancreatin and human pancreatic lipase) to hydrolyze ester forms and its ability to cleave different xanthophyll esters (Breithaupt et al., 2002). Colipase, taurocholate salts and phospholipase A2 were added in order to check and provide optimum conditions for xanthophyll hydrolysis and micellization. Neither oil solutions nor antioxidants (i.e., BHT) were added to the reaction mixture, and factors such as length of time in each phase (up to 2 h in gastric media and up to 4 h in duodenum), type and speed of mixing and isolation procedures of supernatants (aqueous-micellar fractions) (up to 16 h) were also evaluated.

Fruits (loquat [*Eriobotrya japonica*] and orange [*Citrus sinensis*]) were used as sources of xanthophyll esters and broccoli (*Brassica oleracea*) as a source of non-esterified carotenoids and tocopherols. Final conditions were as follows: fruits and vegetables were homogenized with a kitchen blender for 15-second intervals (maximum 4 intervals) to simulate mastication. 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) (EC 3.2.1.1) was 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 (EC 3.4.23.1) was 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 (9 ml, containing bovine bile [0.6 g]) were introduced after neutralization of the pH (7.8), and the human pancreatic lipase (1 unit), colipase

(12.5 μg) (EC 259.490.1), 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. Gastric juice and saliva were prepared the day before the digestion experiment, whereas duodenal solutions were prepared daily. The entire procedure was performed under dimmed light.

Considering that the phytochemical contents in the supernatants and residues reflect the amounts of compounds available in the small and large intestine, respectively (Hedren et al., 2002; Serrano et al., 2005), transfer from the duodenal digesta to the aqueous-micellar phase was estimated by calculating the proportion of phytochemicals in the supernatants. To compare the recovery of free and esterified carotenoids and tocopherols in the supernantants, we performed different experiments using overnight sedimentation and several low-speed centrifugation conditions (5000 rpm for 20 min, 12,900 rpm for 2 h, and 25,000 rpm for 30 min at room temperature) (Model 3K30, Sigma). Overnight decantation rendered the best results, whereas, of the centrifugation protocols, overall, centrifugation at 5000 rpm for 20 min resulted in the best recovery and the more practical conditions. Thus, calculations of transfer from the duodenal digesta to the aqueous-micellar phase (micellization) were estimated using the proportion of phytochemicals in the supernatants both after decantation for 16 h at room temperature and after low speed centrifugation (5000 rpm for 20 min).

2.3. Sample extraction and analysis

Digestion assays with loquat (*Eriobotrya japonica*) ($n = 14$), orange (*Citrus sinensis*) ($n = 7$) and broccoli (*Brassica oleracea*) ($n = 6$) were performed in triplicate, using fresh orange segments ($n = 4$) and orange juice ($n = 3$), canned loquat ($n = 11$) and extracts ($n = 3$), and boiled ($n = 1$) and microwaved broccoli ($n = 5$). Also, an additional tube containing standards of the major carotenoids, tocopherols and retinyl palmitate (control) was evaluated in each assay to assess changes outside the food matrix.

To establish stability to digestion (i.e., recovery and isomeric profile), extent and specificity of ester hydrolysis and micellization, the analysis of carotenoids and tocopherols was performed in samples collected from the starting material and at different time points during digestion (saliva, gastric and duodenal phase) and after transfer into supernatants. 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 (Granado, Olmedilla, Gil-Martínez, & Blanco, 2001; Olmedilla, Granado, Gil-Martínez, Blanco, & Rojas-Hidalgo, 1997). Detection was carried out by a photodiode array (Model 996, Waters Associates, Milford, USA) set at 325 nm for retinoids, 294 nm for tocopherols and 450 and 402 nm for carotenoids. Using this method, retinol, retinyl acetate, retinyl palmitate, α-, γ- and δ-tocopherol, violaxanthin, neoxanthin, *trans*-lutein, zeaxanthin, 13/15-*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 and 13/15-*cis*-β-carotene, phytofluene and phytoene can be simultaneously determined in saponified extracts. Identification of compounds was carried out by comparing retention times with those of authentic standards, on-line UV–VIS spectrum and chemical reactions (i.e., alkaline hydrolysis, reaction with diluted hydrochloric acid).

Within-day variability for major compounds (i.e., lutein, β-cryptoxantin, β-carotene, α- and γ-tocopherols) was on average <10%, although the degree of matrix homogenization affected precision. Enzyme activity was not checked and the manufacturers' specifications were assumed to be reliable, and fresh matrices were always used due to the instability of matrix components (i.e., ascorbic acid).

2.4. Calculations

In order to enable the comparison of the results from different experiments and matrices, the parameters evaluated (i.e., stability, extent of hydrolysis, isomerization) were expressed as percentages of the initial total content of carotenoids and tocopherols in the food. Then, the percentages from different assays were studied by pooling data from all experiments and were compared according to other variables (i.e., food item, type of matrix, enzyme amount), and descriptive statistics were used (mean, median, $CI_{95\%}$, etc.). β-cryptoxanthin, the characteristic provitamin A xanthophyll in both fruits, was considered as the reference item for the evaluation of hydrolysis. The results were interpreted on the basis of data from crude and saponified extracts.

3. Results

3.1. Efficacy of hydrolysis

The digestion conditions were established considering the maximum hydrolysis of xanthophyll esters and of retinyl ester (as a control). During the saliva and gastric phase, no significant hydrolysis of xanthophyll esters or retinyl palmitate was observed and prolonging the time in gastric conditions apparently led to lower recovery of free β-cryptoxanthin. During the duodenal phase of the entire simulated digestion, in the presence of human pancreatic lipase, the increase in free β-cryptoxanthin was <8% suggesting no relevant xanthophyll ester hydrolysis (Fig. 1), even when complete hydrolysis of retinyl palmitate was obtained during the first hour. The addition of cholesterol esterase led to an increase in free xanthophylls, the levels of which were higher during the first hour and increased during the second, reaching a plateau with minor, if any, increments after longer incubation times (Fig. 2). Hydrolysis of xanthophyll esters was incomplete,

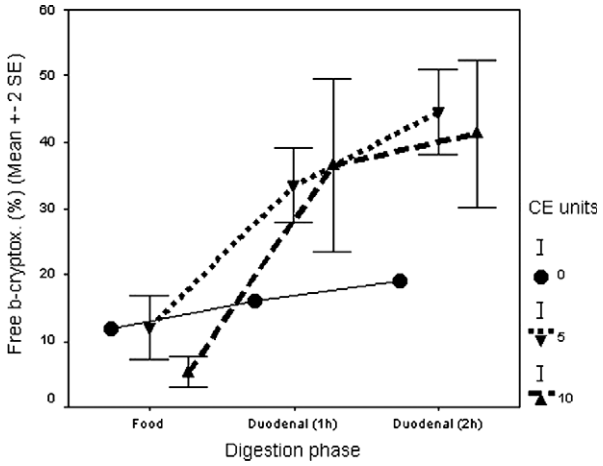

Fig. 1. Effect of the presence and amount of enzyme(s) in the media on the rate of β-cryptoxanthin ester hydrolysis. Human pancreatic lipase is present in all experiments (1 unit). CE, Cholesteryl esterase.

regardless of the lot-to-lot variability in enzyme activity, complexity of ester profile (i.e., loquat versus orange), substrate-to-enzyme ratio, time of duodenal incubation and addition of other components (i.e., colipase, phospholipase A_2, taurocholate salts).

Total content of individual carotenoids and tocopherols and percentage of nonesterified forms present in the foods and at the end of the digestion (gastric plus duodenal phase) are shown in Table 1. At the end of the duodenal phase, the mean rate of "net" hydrolysis of β-cryptoxanthin esters (total free minus free β-cryptoxanthin initially present in the food) was 27% ($CI_{95\%}$ 22–32%) of the total amount present in the fruits (loquat and orange combined), whereas for lutein and zeaxanthin (in orange), the "net" hydrolysis was, on average, lower, 6% and 18%, respectively. Overall, differences were observed depending on the type of fruit (loquat versus orange) and the matrix assayed both for the "net" hydrolysis of β-cryptoxanthin ($p = 0.07$) and the total free β-cryptoxanthin recovered in the final digesta ($p < 0.06$). The degree of "net" hydrolysis was 36% ($CI_{95\%}$ 22–51%) for orange juice, 20% ($CI_{95\%}$ 7–34%) for orange segments, 32% ($CI_{95\%}$ 27–38%) for extracted loquat and 23% ($CI_{95\%}$ 14–33%) for homogenized canned loquat. The amounts of other free xanthophylls, tentatively identified as α-cryptoxanthin, neoxanthin and violaxanthin, although not quantified, also increased during the duodenal phase.

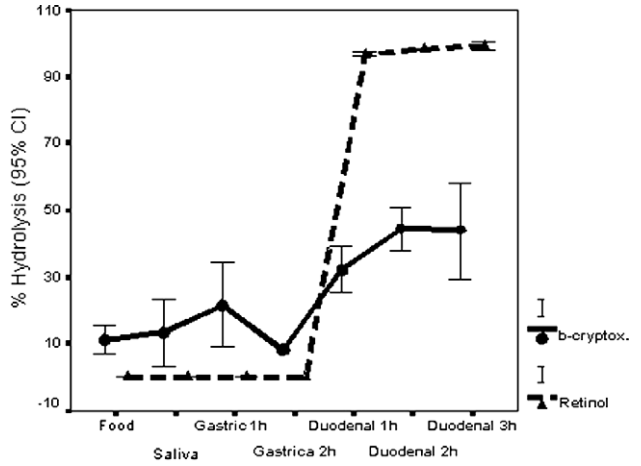

Fig. 2. Percentage of free β-cryptoxanthin and retinol (standard) recovered at different stages of the *in vitro* digestion protocol in the presence of cholesterol esterase in the media (5 units).

3.2. Stability to digestion and transfer into supernatants

In fruits, the chromatographic profile of the final digesta reflected the initial content except that more free xanthophyll and less xanthophyll esters were present. For epoxy carotenoids (i.e., neoxanthin, violaxanthin), a substantial decrease was observed during the gastric phase, but new peaks appeared during the duodenal phase that, based on UV–VIS changes after chemical reactions, were interpreted as losses of epoxy xanthophylls due to the acidic conditions, rearrangements of 5–6 to 5–8-epoxy groups and hydrolysis of ester forms after epoxy group rearrangements. Also, the percentages of *cis*-lutein and *cis*-β-carotene in processed broccoli (mean ($CI_{95\%}$); 7% (6%, 8%), 18% (17%, 19%), for *cis*-lutein and *cis*-β-carotene, respectively) did not increase consistently during saliva, gastric or duodenal phase (mean, ($CI_{95\%}$); 6% (6%, 7%), 19% (15%, 23%), for *cis*-lutein and *cis*-β-carotene, respectively), even when, under acidic conditions, formation of 13/15-*cis*-lutein was observed in the control solution.

Considering all the assays in fruits, recovery of β-cryptoxanthin (based on the content in saponified extracts in each phase) was 72% ($IC_{95\%}$ 59–84%) of the initial content, suggesting that, on average, about 25% of the β-cryptoxanthin is lost during digestion. Recovery (median) reached ca. 85–90% for lutein and zeaxanthin in orange, and 75% ($IC_{95\%}$ 63–87%) for β-carotene in loquat. In broccoli, up to 90% of lutein, β-carotene and γ-tocopherol and 77% of α-tocopherol were recovered at the end of the simulated digestion. Free β-cryptoxanthin represented, on average, 38% ($IC_{95\%}$, 32–44%) of the total initial content (combining data from loquat and orange) but, considering the final recovery of β-cryptoxanthin (stability), that means that about 50% of the β-cryptoxanthin present during this phase continued to correspond to the free form (Fig. 3). Similarly, at the end of the duodenal phase, free forms of lutein and zeaxanthin accounted for 26% and 43%, respectively, of the amounts present initially (Table 1).

Incorporation of phytochemicals into the supernatants (micellization) differed depending on the protocol used. In fruits, the amounts of free forms of lutein, zeaxanthin and β-cryptoxanthin, β-carotene and α-tocopherol recovered were almost 2-fold greater after overnight sedimentation of the digesta (16 h, room temperature, protected from light) than those obtained with any low-speed centrifugation procedure (5000 rpm for 20 min, room temperature), whereas in broccoli, no differences were observed between the two methods for any of the analytes assayed.

Table 1
Total content and percentage of free forms (mean, 95% CI) of the major carotenoids and tocopherols in the foods assayed[a]

Food	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene	α-Tocopherol	γ-Tocopherol
Loquat (n = 14)[b]						
Content in food (μg/100 g)	–[c]	–	518 (371–666)	207 (138–276)	–	–
% As free forms in food	–	–	6 (4–8)	NA[d]	–	–
% as free forms in duodenal phase	–	–	32 (23–40)	NA[d]	–	–
Orange (n = 7)						
Content in food (μg/100 g)	31 (13–49)	18 (2–34)	45 (28–63)	–	198 (−87 to 483)	–
% as free forms in food	20 (5–45)	26 (6–45)	18 (9–27)	–	NA[d]	–
% as free forms in duodenal phase	26 (1–51)	43 (37–50)	45 (39–52)	–	NA[d]	–
Broccoli (n = 6)						
Content in food (μg/100 g)	1281 (864–1698)	–	–	898 (615–1181)	2718 (1509–3926)	618 (209–1028)
Content in duodenal phase (μg/100 g)	892 (752–1034)	–	–	685 (75–1022)	2035 (1285–2784)	471 (380–562)

[a] Total content was determined in saponified extracts. Free forms of xanthophylls were quantified in unsaponified extracts and referred to the total initial content in saponified extracts.
[b] Number of assays performed in triplicate.
[c] Not determined.
[d] NA, not applicable.

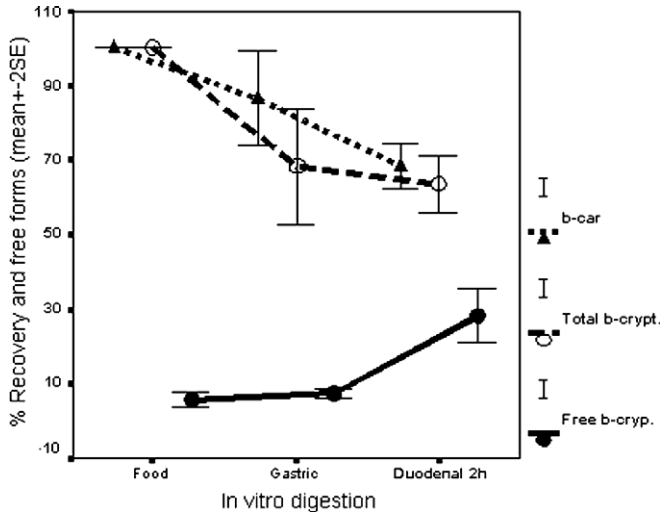

Fig. 3. Stability and extent of hydrolysis of major carotenoids at the end of the *in vitro* digestion (loquat). For final digestion conditions, see Section 2.

Compared to the initial contents in foods, after 16 h of decantation, the amounts (medians) in the supernantants represented 39% of free β-cryptoxanthin, 42% of free zeaxanthin, 27% of free lutein (<10% from broccoli), 17% of β-carotene, and 14% and 17% of γ-tocopherol and α-tocopherol, respectively. Considering the content in the duodenal phase, xanthophylls were more efficiently transferred to the supernatant than carotenes and tocopherols, and *cis*-carotenoid levels in the micellar fraction were also higher, both for 13/15-*cis*-β-carotene (20–27%) and *cis*-lutein in broccoli (ca. 12% versus 6% in the duodenal phase). Qualitatively, other xanthophylls (i.e., neoxanthin, violaxanthin) and carotenes (i.e., phytoene), as well as ester forms, were also observed in the supernatants, regardless of the micellization protocol used.

4. Discussion

To assess the bioaccessibility of carotenoids, including xanthophyll esters, and tocopherols from foods, we optimized a validated approach, contrasted against both static and dynamic gastrointestinal models in a multi-lab comparison study (Oomen et al., 2002). Coinciding with previous studies (Chitchumroonchokchai et al., 2004; Garret et al., 1999; Serrano et al., 2005), we found that more than 70% of the carotenoids and tocopherols remained in the final digesta and thus, available for micellization, although in fruits, this estimate refers to the presence of free and ester forms of xanthophylls.

Cholesterol esterase was needed for the hydrolysis of xanthophyll esters present in fruits, regardless of the acyl group and the xanthophyll involved, suggesting that the enzyme effectively cleaved different natural esters, as previously observed (Breithaupt et al., 2002; Breithaupt et al., 2003; Jacobs, LeBoeuf, McCommas, & Tauber, 1982). Nevertheless, hydrolysis was incomplete, as observed in enzymatic assays (Breithaupt et al., 2002) and, on average, the amount of β-cryptoxanthin esters hydrolyzed (i.e., loquat and orange) was ca. 40% of the total initial β-cryptoxanthin content. Considering the recovery obtained at this stage, this indicates that ca. 50% of the β-cryptoxanthin present in the duodenum corresponds to the free form. Although this percentage is somewhat lower than those reported for papaya and loquat oleoresins in enzymatic assays (ca. 60% at 1 h) (Breithaupt et al., 2002), it is very close to that recently reported for other xanthophyll ester-containing foods (Chitchumroonchokchai & Failla, 2006). In addition, in the present study, enzyme activity was not measured, a fact that may have reduced the yield in the assays (Breithaupt et al., 2002). In addition, a matrix effect was clearly present, since variations in neither the substrate-to-enzyme ratio nor the time allowed for the reaction affected the extent of hydrolysis, whereas higher amounts of free forms (hydrolysis) were obtained when

juices and extracts were assayed, suggesting that the solubilisation of the substrates (i.e., ester forms) from the matrix into the media is a critical factor for efficient hydrolysis.

4.1. Transfer into supernatants

The transfer from the food matrix into micelles is one of the steps that determines the extent of carotenoid absorption from different foods, and influencing factors include type of matrix, particle size, hydrophobicity of the carotenoid, pH of the gastric phase, soluble and insoluble indigestible fractions, taurocholate concentration, amount and type of lipids, phospholipids and bile salt content, as well as methodological issues (i.e., centrifugation) (Hedren et al., 2002; Jacobs et al., 1982; Tyssandier et al., 2003; Failla & Chitchumroonchokchai, 2005; Serrano et al., 2005). For this step, we used overnight sedimentation as the reference protocol, based on preliminary results in our laboratory and previous reports involving ultracentrifugation and low speed centrifugation (Garret et al., 1999; Hedren et al., 2002; Oomen et al., 2003). This reference approach was also consistent with physiological events, such as the estimated transit time through the stomach and small intestine (fed conditions, up to 12–14 h); the time-dependence of carotenoid uptake by Caco-2 cells, requiring up to 16 h; the peak maxima obtained in blood after a single ingestion of carotenes and xanthophylls (<12 h) (Chitchumroonchokchai et al., 2004; Breithaupt et al., 2003; During et al., 2002; Failla & Chitchumroonchokchai, 2005; Rich, Faulks, Wickham, & Filleery-travis, 2003; Wingerath, Stahl, & Sies, 1995) and observations in humans, in whom a longer food transit time is related to a higher lutein absorption, indicating that time is a much more crucial controlling factor during transfer to lipid phase for lutein than for β-carotene (Faulks, Hart, Brett, Dainty, & Southon, 2004).

Micellization of lutein and zeaxanthin has been reported to be more efficient than that of β-carotene or lycopene (Chitchumroonchokchai et al., 2004; Garret et al., 1999; Tyssandier et al., 2003), coinciding with the preferential incorporation into chylomicrons and the greater absorption of lutein and zeaxanthin reported in humans (Gartner, Stahl, & Sies, 1996; Van Het Hof et al., 1999). In this respect, it has been suggested that transfer to the micelles is inversely proportional to the hydrophobicity of the carotenoid (Failla & Chitchumroonchokchai, 2005), and our results agree with these observations in that the amounts of xanthophylls (i.e., lutein, zeaxanthin and β-cryptoxanthin) recovered in supernatants were greater than those of carotenes or tocopherols (regardless of the method used).

However, the rate of incorporation into supernatants observed in the present study (zeaxanthin = β-cryptoxanthin > lutein > tocopherols = β-carotene) suggests that hydrophobicity is not the only controlling factor since the transfer of β-cryptoxanthin was greater than that of lutein or tocopherols. Interestingly, on average, the amount of free β-cryptoxanthin transferred was almost 3-fold that observed for β-carotene, both in loquat and broccoli, a fact that could be related to the higher efficiency of orange juice for raising serum retinol concentrations, compared to that of green vegetables reported in humans (De Pee et al., 1998). Regarding tocopherols, under the assay conditions, α- and γ-tocopherol showed a similar rate of incorporation into supernatants, a finding consistent with the similar apparent efficiency of intestinal absorption in humans (Traber, Cohn, & Muller, 1993). Moreover, transfer efficiency varies according to the type of vegetable (Failla & Chitchumroonchokchai, 2005), and our results also support this observation since lutein from orange was incorporated to a greater extent than that from broccoli, supporting a matrix effect, as previously reported (Chitchumroonchokchai & Failla, 2006; Failla & Chitchumroonchokchai, 2005; Serrano et al., 2005). Similarly, the proportions of *cis*-β-carotene and *cis*-lutein were higher in the supernatants than in the duodenal digesta, coinciding with a preferential incorporation and/or possible isomerization in the aqueous phase during decantation (Failla & Chitchumroonchokchai, 2005; Tyssandier et al., 2003).

Peaks tentatively identified as free epoxy-carotenoids and metabolites, as well as xanthophyll esters, were also found in the supernatants when different micellization protocols, including ultracentrifugation, were used (unpublished observations). Although xanthophyll epoxides have not been detected in humans after a single ingestion (Barua & Olson, 2001), their transfer into the aqueous phase is not surprising since studies in Caco-2 cells and rats have shown that both these xanthophylls and their 5–8-epoxy metabolites produced during digestion are absorbed and distributed to specific tissues (Asai, Terasaki, & Nagao, 2004; Sugawara et al., 2001).

The presence of ester forms in the aqueous-micellar phase after *in vitro* digestion has just been reported (Chitchumroonchokchai & Failla, 2006), confirming the present findings observed using different micellization protocols, i.e., overnight sedimentation, low-speed and ultracentrifugation. Although this may simply be a consequence of their hydrophobicity and partition behaviour, given the absence of xanthophyll esters during the postprandial state in humans (Breithaupt et al., 2003; Granado, Olmedilla, Gil-Martínez, & Blanco, 1998; Wingerath et al., 1995), it could be that micellized ester forms are cleaved at the brush border membrane of the intestine, as reported for retinyl palmitate in rats and humans (Rigtup, McEwen, Said, & Ong, 1994), or within the enterocyte. If so, the amount of xanthophylls potentially available for absorption would be higher and interactions during micellization and absorption could occur, affecting the bioavailability of xanthophyll ester-containing fruits, including their provitamin A value.

Finally, comparison of *in vitro* digestion models is complicated since, although they are based on human gastrointestinal conditions, it is usually difficult to ascertain which one provides the most accurate bioaccessibility values in terms of the human situation unless they can be compared with in vivo studies (Oomen et al., 2002). In this respect,

the present protocol, although cannot be considered as fully physiological (i.e., mastication, gastric pH), provides estimations, both qualitative and quantitative, for a wide range of carotenoids and tocopherols that are consistent with observations in other *in vitro* digestion models and human studies.

Acknowledgements

The support of the Ministerio de Ciencia y Tecnología (AGL 2002-04059-C02-01) and Instituto de Salud Carlos III (RCMN C03/08) is acknowledged. We are also indebted to Martha Messman for preparing the manuscript.

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