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Bioavailability of carotenoids and α-tocopherol from fruit juices in the presence of absorption modifiers: *in vitro* and *in vivo* assessment

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The food industry is playing an increasing role in the development and marketing of new products although little is known regarding the bioavailability of the phytochemicals they contain. Our aim was to assess the effect of the presence of absorption modifiers (milk and iron) on the *in vitro* bioaccessibility and the serum response *in vivo* of carotenoids and α-tocopherol from fruit juices. Thirty-two young women participated in a three-period (21 d each) supplementation study with a 2-week wash-out in between. Subjects consumed consecutively 2 × 250 ml/d vitamin C-fortified juices supplied as fruit juice, fruit juice containing milk and fruit juice containing milk and iron. Fasting blood samples were collected before and after each supplementation period. *In vitro* bioaccessibility of carotenoids and α-tocopherol was assessed by a static digestion model. Vitamin E and carotenoids from both studies were determined by HPLC. *In vitro*, xanthophyll ester hydrolysis and transference of free xanthophylls and α-tocopherol into the micellar phase were higher in the presence of absorption modifiers. *In vivo*, consumption of the fruit juices provoked significant increments (within-subject) of α-tocopherol and some carotenoids in serum. Dose-adjusted increments in serum of some carotenoids were higher when subjects consumed juices with milk and milk plus iron, although differences did not reach statistical significance. In conclusion, the presence of milk and milk plus iron do not influence the bioavailability of carotenoids and α-tocopherol from fruit juices *in vivo*. Our results support the use of *in vitro* models to assess food-related factors affecting bioavailability of carotenoids and tocopherols from foods.

Bioavailability: *In vitro* digestion: Carotenoids: Human studies

Fruits and vegetables are major sources of biologically active compounds, many of which, i.e. carotenoids and tocopherols, may have beneficial effects against chronic diseases[1]. To achieve the benefits associated with the consumption of fruits and vegetables, different approaches are considered to increase the content and/or the bioavailability of these components including agricultural practices, biotechnology and food technology[2].

Bioavailability is a critical feature in the assessment of the role of phytochemicals in human health and approaches to the study of the bioavailability of food components include *in vitro* and *in vivo* methods. *In vitro* models based on human physiology have been developed as simple, inexpensive, non-invasive 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, tocopherols and polyphenols)[3–6]. Nevertheless, since several factors are capable of influencing vitamin bioavailability at different points, *in vitro* methodology for bioavailability assessment and its potential predictive value regarding human absorption of phytochemicals should be validated in different *in vivo* situations[7].

Different dietary factors are known to affect the bioavailability of carotenoids and tocopherols in man[8,9]. Both the amount and type of fat affect the bioavailability of some (i.e. lutein), but not all, carotenoids[10,11], while plant sterols and water-soluble fibres (i.e. pectin) decrease the absorption of β-carotene, lycopene, lutein and tocopherols in man[12]. Similarly, *in vitro* bioaccessibility studies of carotenoids have also shown positive and negative associations with fat and fibre content[13,14]. In this context, the transfer into micelles constitutes a key step of carotenoid absorption from different foods, and different influencing factors have been reported including soluble and insoluble indigestible fractions, taurocholate amount, amount and type of lipids and acyl moieties, the content of Klason lignin and NSP, phospholipids and bile salt content[5,15–19].

Currently, the food industry is playing an increasing role in the development and marketing of new products and the addition of milk to fruit-based juices and beverages is becoming a common practice. Several components simultaneously present in foods, such as carotenoids, α-tocopherol and food-derived peptides (such as casein phosphopeptides, generated from casein digestion of milk) are bioactive, displaying antioxidant activities[20–22] and perhaps interacting

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by different mechanisms during digestion and absorption. In this sense, milk-derived peptides may inhibit lipid oxidation by chelation and non-chelating mechanisms[23,24], show cytomodulatory and inmunomodulatory effects and may bind minerals (i.e. calcium, iron, zinc)[22,25,26], increasing their solubility and displaying a positive effect on mineral absorption (i.e. calcium, iron), although conflicting results have been reported[27]. Thus, because of their potential beneficial effect on mineral bioavailability and other biologically relevant activities that they may display, food-derived peptides are considered as potential functional ingredients in foods[22,28]. However, while this may be relevant for the food quality and stability of micronutrients in foods, little is known regarding the effects on the bioavailability of phytochemicals. Thus, to understand better the effect of the presence of absorption modifiers (milk-derived peptides and iron) on the bioavailability of carotenoids and α-tocopherol, we used a complementary approach; an *in vitro* model to assess pre-absorptive events (i.e. stability, ester hydrolysis, isomerization, micellarization; bioaccessibility) and an *in vivo* study to evaluate the serum response upon regular consumption of the fruit juices (bioavailability).

Subjects and methods

Subjects

Thirty-two young women (20–30 years) participated in a three-period (21 d each) supplementation study with a 2-week wash-out in between. Qualitative and quantitative content of the juices was approached, based on industrial and scientific reasons, and the final product specifically developed for the purpose of the present study (Hero S.A., not commercially available). Juices were prepared according to the protocols and controls required by the law and the adequacy for human consumption, as required by the Comité Etico de Investigación Clínica, was certified. Because of manufacturing and logistic (storage) limitations, participants could not be randomly assigned at the start and thus, subjects consumed consecutively 2 × 250 ml/d vitamin C-fortified juices supplied as fruit juice, fruit juice containing milk (skimmed milk powder) and fruit juice containing milk and iron (Table 1). In addition, because of these constraints, juices had to be prepared on different occasions so that the carotenoid content could be eventually not equivalent (especially for the juices containing milk and iron), probably due to a different ripeness degree of the fruits used.

All participants were required to have biochemical profile and serum levels of vitamins A and E and carotenoids within accepted reference ranges, whereas exclusion criteria included the use of vitamin and/or herbal supplements, dieting, chronic medication, or intercurrent disease or infection that could alter the bioavailability or the status of the compounds of interest. No other changes were made in the diet or lifestyle of the participants except that they should avoid the consumption of the fruits, or similar, contained in the juices (i.e. orange, mandarin, grapes, peach, apricot). Subjects were asked to keep a record of their diets to check the compliance with these dietary recommendations.

Overnight fasting blood samples were collected before and after each supplementation period for analysis of vitamin E and carotenoids. The study protocol was approved by the Comité Etico de Investigación Clínica of the Hospital Universitario Puerta de Hierro, and all subjects were informed and gave their signed consent.

Standards and reagents

Unless otherwise stated, all reagents and materials used in the *in vitro* protocol and analysis of blood samples for vitamins A and E and carotenoids were purchased from Sigma (Madrid, Spain), VWR Internacional Eurolab (Mollet del Vallés, Spain) and Carlo Erba (Madrid, Spain). Zeaxanthin was generously supplied by DMS (formerly Hoffmann-La Roche, Basel, Switzerland).

In vitro *digestion*

To assess the *in vitro* bioaccessibility of carotenoids and α-tocopherol from fruit juices in the presence of absorption modifiers, a static gastrointestinal digestion model previously applied to fruits and vegetables was used[29]. At each step of the digestion (food, gastric, duodenal and micellar), aliquots (1 ml) were collected in duplicate, extracted before and after chemical (KOH) hydrolysis, and analysed by HPLC[30].

Phytochemical analysis

For vitamins A, E (α-tocopherol) and carotenoid analysis in serum, samples were processed as described elsewhere[30]. Briefly, 0·5 ml serum was mixed with 0·5 ml ethanol containing internal standard (retinyl acetate), vortexed, and extracted twice with 2 ml methylene chloride–hexane (1:5). Organic phases were pooled, evaporated to dryness, and reconstituted to be injected (tetrahydrofuran–ethanol) on to the high-performance liquid chromatograph. The chromatographic system consisted

Table 1. Composition of fruit juices (FJ) used in the human study*

Type of juice	Lutein (μg/100 ml)	Zeaxanthin (μg/100 ml)	β-Cryptoxanthin (μg/100 ml)	β-Carotene (*trans*-) (μg/100 ml)	α-Tocopherol (μg/100 ml)	L-Ascorbic acid (mg/100 ml)†	Milk (%)†	Iron (mg/100 ml)†
FJ	30	70	72	58	477	0·054	None	None
FJ+milk	35	60	71	53	484	0·054	11 %	None
FJ+milk+iron	18	18	38	38	360	0·054	11 %	3 mg

* Orange, peach, grape juice; carotenoid and α-tocopherol content in saponified extracts.
† Information supplied by the manufacturer (Hero S.A.); percentage of milk in the final product; iron sulphate (II) added to the product.

of a Spheri-5-ODS column (Applied Biosystems, San Jose, CA, USA) with gradient elution of acetonitrile–methanol (85:15) for 5 min to acetonitrile–methylene chloride–methanol (70:20:10) for 20 min. Ammonium acetate (0·025 M) was added to the methanol. Detection was carried out by a photodiode array (Model 2996; Waters Associates, Milford, MA, USA) set at 294 nm for tocopherols and 450 nm for carotenoids. Using this method, α-tocopherol, *trans*-lutein, zeaxanthin, 13/15-*cis*-lutein, α-carotene, all-*trans*-β-carotene, 9-*cis*-β-carotene and 13/15-*cis*-β-carotene, among other carotenoids, can be determined simultaneously. Identification of compounds was carried out by comparing retention times with those of authentic standards and on-line UV–visible spectra.

Samples from each individual (obtained before and after the intervention) were analysed on the same day to reduce analytical variability. The short- and long-term precision and accuracy of the analytical method was verified periodically through our participation in the Fat-Soluble Quality Assurance Program conducted by the National Institute of Standards and Technology (Gaithersburg, MD, USA).

Statistics

In order to achieve consistency in the results from the different *in vitro* experiments, the parameters evaluated (i.e. degree of hydrolysis) were expressed as percentages of the total amounts initially present in the juices (saponified extracts), and descriptive statistics were used (means and 95 % CI). *In vitro* results were interpreted on the basis of data from crude and saponified extracts. For *in vivo* studies, since most of the parameters showed normal distributions, differences in serum concentrations at baseline and at the end of each intervention period, within-subject variation due to the juice consumption (paired data) and differences due to the type of juice were assessed using parametric methods (ANOVA and *post hoc* Scheffé test, paired *t* test). However, because some carotenoid distributions did not fit the normality criteria, comparisons were also performed using non-parametric tests (Wilcoxon signed ranks and Kruskal–Wallis test). Statistical significance was set at $P<0·05$, and the calculations were performed with SPSS version 8.0 statistical software for Windows (SPSS Inc., Chicago, IL, USA).

Results

In vitro *bioaccessibility*

Overall, compared to fruit juice (reference), the extent of xanthophyll ester hydrolysis (i.e. β-cryptoxanthin, lutein, zeaxanthin) and the amount of free xanthophylls and α-tocopherol transferred into the micellar phase was only significantly higher in the presence of milk (Table 2).

In vivo *bioavailability*

Baseline serum levels of carotenoids, α-tocopherol and retinol were not different at the start of each intervention period (Table 3). Regardless of the type of juice consumed, consumption of each type of fruit juice provoked significant increments (within-subject) in the serum levels of α-tocopherol and most, but not all, of the carotenoids provided (Table 4), although the degree of the changes (net increments) varied according to the type of juice consumed, as expected given the different carotenoid and tocopherol content in the juices (see Table 1). However, dose-adjusted increments (net increment divided by the content in 100 ml juice) of carotenoids in serum were not significantly higher when subjects consumed juices with milk and milk+iron (Fig. 1; Table 4).

Discussion

Food and host-related factors influence carotenoid and tocopherol bioavailability[8,9]. Recently, the release of carotenoids from the food matrix, the solubility, the measurement and interpretation of plasma response, and the differences in inter-individual response have been referred to as the challenges to understanding and measuring carotenoid bioavailability[31]. To cover all these aspects, in the present study we use a complementary approach to assess

Table 2. Ester hydrolysis (% of free forms in the duodenal phase) and bioaccessibility (% of free forms in the micellar phase) of carotenoids and α-tocopherol under *in vitro* conditions†

(Mean values and 95 % confidence intervals)

	Lutein (%)		Zeaxanthin (%)		β-Cryptoxanthin (%)		*Trans*-β-carotene (%)		α-Tocopherol (%)‡	
Type of juice	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI
FJ§	100		100		100		100		100	
Duodenal phase‖	29	11, 47	19	14, 24	34	31, 36	101	89, 114	89	78, 100
Micellar phase‖	26	18, 33	18	14, 22	30	22, 38	57	43, 71	84	70, 98
FJ+milk§	100		100		100		100		100	
Duodenal phase‖	32	29, 36	31*	27, 35	51*	46, 56	106	95, 117	116	89, 140
Micellar phase‖	41*	28, 55	39*	24, 55	55*	35, 75	75	38, 111	110*	73, 145
FJ+milk+iron§	100		100		100		100		100	
Duodenal phase‖	29	22, 37	28*	20, 35	36	28, 45	85	51, 118	99	47, 150
Micellar phase‖	29	14, 44	25	6, 44	38	14, 63	59	47, 70	98	89, 107

FJ, fruit juice.
Mean values were significantly different from those of the FJ group (ANOVA and Scheffé *post hoc* test): *$P<0·05$.
† For details of procedures and diets, see the Subjects and methods section and Table 1.
‡ For α-tocopherol, values referred to the percentage of recovery in each digestion phase (no ester forms were present).
§ Total content in saponified extracts (used as reference values = 100 %).
‖ Percentage of free forms (unsaponified extracts) against total content (saponified extracts).

Table 3. Serum levels of carotenoids and α-tocopherol at the start of each intervention period (*n* 32)*
(Mean values and 95 % confidence intervals)

	Lutein (μmol/l)		Zeaxanthin (μmol/l)		β-Cryptoxanthin (μmol/l)		*Trans*-β-carotene (μmol/l)		α-Tocopherol (μmol/l)		Retinol (μmol/l)	
Type of juice	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI
FJ	0·23	0·19, 0·26	0·04	0·03, 0·05	0·43	0·31, 0·56	0·34	0·26, 0·43	20·9	19·6, 22·2	1·64	1·47, 1·78
FJ+milk	0·25	0·21, 0·30	0·05	0·04, 0·06	0·38	0·31, 0·43	0·34	0·26, 0·39	21·5	19·9, 23·0	1·75	1·61, 1·92
FJ+milk+iron	0·26	0·21, 0·30	0·06	0·04, 0·07	0·36	0·31, 0·40	0·34	0·28, 0·41	21·8	20·5, 23·1	1·75	1·57, 1·89
Differences between baseline levels†	NS		NS		NS		NS		NS		NS	

FJ, fruit juice.
* For details of procedures and diets, see the Subjects and methods section and Table 1.
† ANOVA and Scheffé *post hoc* test.

Table 4. Within-subject net increments (final minus basal) in serum levels of carotenoids and α-tocopherol upon consumption of fruit juices (FJ)‡
(Mean values and 95 % confidence intervals)

	Lutein (μmol/l)		Zeaxanthin (μmol/l)		β-Cryptoxanthin (μmol/l)		Total β-carotene (μmol/l)		α-Tocopherol (μmol/l)	
Type of juice	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI
FJ	+0·012	−0·014, 0·038	+0·060*†	0·044, 0·076	+0·123*†	0·067, 0·179	+0·060*†	0·022, 0·097	1·65*†	0·23, 3·04
FJ+milk	+0·009	−0·014, 0·032	+0·062*†	0·049, 0·076	+0·147*†	0·109, 0·184	+0·052*†	0·022, 0·082	+0·72†	−0·28, 1·70
FJ+milk+iron	+0·004	−0·012, 0·019	+0·021*†	0·011, 0·032	+0·090*†	0·058, 0·123	+0·019	−0·015, 0·054	+1·04*†	0·51, 1·58
Differences in the increments (dose-adjusted) according to the type of juice§	NS		NS		NS		NS		NS	

Mean values were significantly different from those of the FJ group (paired *t* test): *$P<0·05$.
Mean values were significantly different from those of the FJ group (Wilcoxon signed ranks test): †$P<0·05$.
‡ For details of procedures and diets, see the Subjects and methods section and Table 1.
§ ANOVA, Dunnett *post hoc* test and Kruskal–Wallis test.

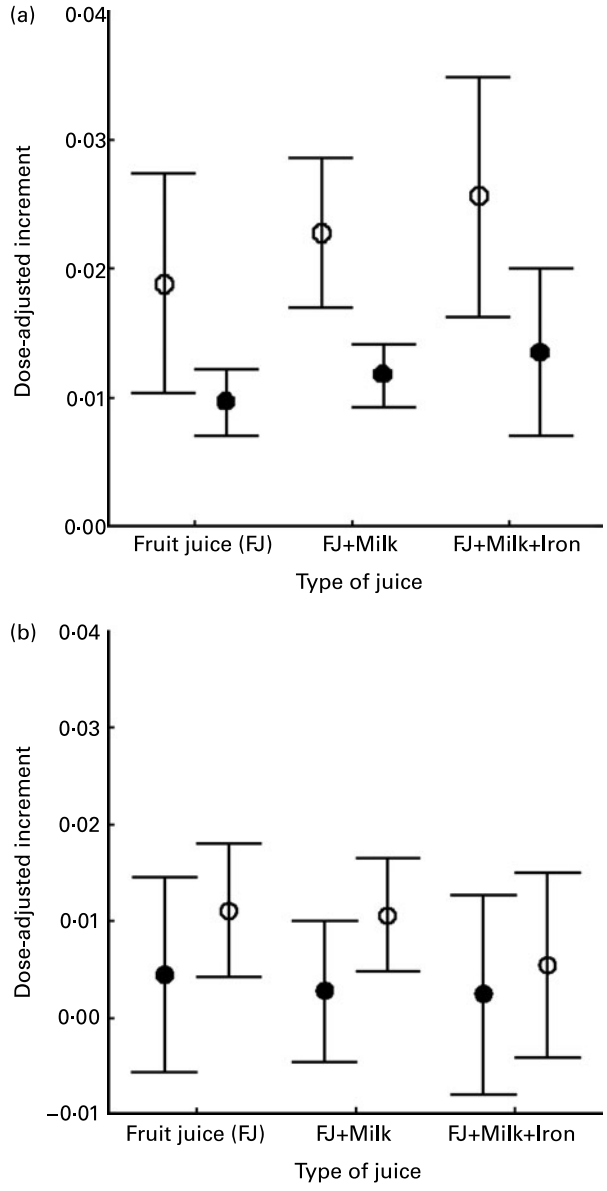

Fig. 1. Dose-adjusted increments in serum of β-cryptoxanthin (●) and zeaxanthin (○) (a), and β-carotene (○) and lutein (●) (b), after regular consumption (21 d) of each type of fruit-based juice. Values are means with 95 % CI depicted by vertical bars.

the effect of absorption modifiers (milk-derived peptides and iron) on the bioaccessibility (*in vitro*) and bioavailability (*in vivo* response) of carotenoids and α-tocopherol from fruit juices.

In vitro methods may be appropriate for studying pre-absorptive processes, although their validity as an index of absorbability and/or bioavailability should be validated in different *in vivo* situations[5,7]. In the present study, *in vitro* data showed that the degree of ester hydrolysis of xanthophylls (i.e. β-cryptoxanthin and zeaxanthin) increased in the presence of milk, an effect that is also observed during the transference into the micellar phase. Since the presence of free forms in the micellar phase may be indicative of the maximum amount available for absorption[3,5,9,29] based on the present results, a higher serum response after consumption of juices with modifiers could be expected. However, in the *in vivo* study, regular consumption of these juices provoked significant increments (within-subject) regardless of the type of juice consumed, suggesting that the presence of milk and milk plus iron (at the levels used in the present study) did not compromise the bioavailability of carotenoids and α-tocopherol from fruit juices. Moreover, although the presence of milk appeared to improve the serum response for some (i.e. β-cryptoxanthin) but not all carotenoids, this enhancing effect was not significant and thus, the agreement between *in vitro* and *in vivo* results should be considered suggestive but not fully concordant. On the contrary, for α-tocopherol, the *in vitro* and *in vivo* results were inconsistent since the juice with the apparently higher *in vitro* bioaccessibility (fruit juice with milk) showed the lowest serum response *in vivo*. Nevertheless, the present results agree with previous observations regarding the variability of *in vitro* bioaccessibility for different carotenoids in a given food[14,32] and concerning the different behaviour of distinct carotenoids and tocopherols in a food under *in vitro* and *in vivo* conditions[6,33].

It has been suggested that casein phosphopeptides could inhibit lipid oxidation by chelation and non-chelating mechanisms such as free radical scavenging[23,24]. In the present study, although the magnitude of effect observed may be of little relevance, it appears to be associated with the presence of milk and/or milk-derived peptides rather than related to antioxidant mechanisms[22] since the three types of juice contained the same amounts of ascorbic acid. Also, it is known that the presence of fat may improve bioavailability through different mechanisms including digestive enzyme activities and micelles formation[5,8]. However, in the present study, the type of milk incorporated into the juices was fat-free milk and thus differences in the amount of fat, if any, should be negligible. Also, it has been reported that milk caseins stabilize calcium and phosphate ions and that tryptic digestion of casein yields casein phosphopeptides that may interact between caseins and calcium phosphate in the formation of casein micelles, thus enhancing mineral bioavailability[22]. Thus, although we have not assessed casein phosphopeptides under the conditions assayed, a beneficial physico-chemical effect in the system (i.e. mineral chelation, phosphate-induced negative charges and effect on solubility and micelles surface, stabilization of micelles) cannot be ruled out.

Finally, the predictive value of the *in vitro* models relies on the consistency between the results under simulated conditions and those observed *in vivo* under different physiological conditions[4,5,7], although comparisons are often difficult since both approaches lack standardization parameters or traceability to reference methods[34]. In the present study, *in vitro* and *in vivo* results appear to be not consistent since under *in vivo* conditions the enhancing effect of absorption modifiers on carotenoid bioavailability did not reach statistical significance. *In vivo* (human) data constitute the reference standard ('true response') while *in vitro* methods are used as surrogates for prediction. However, we should be aware that the *in vivo* (human) model also displays its own methodological and biological constraints and uncertainties (i.e. first-pass metabolism, intracellular regulation, homeostatic control). For instance,

dose-adjusted responses may compensate the different intake of some compounds but this approach cannot cancel out a different relative contribution within a given diet or the timing of the intervention. Thus, a lack of agreement may not necessarily mean inadequacy of the *in vitro* approach since a full concordance between *in vitro* data and *in vivo* response may not be expected[6,33].

In conclusion, the presence of milk or milk-derived peptides (i.e. casein phosphopeptides) and milk plus iron in fruit juices do not influence the bioavailability of carotenoids and α-tocopherol from fruit juices. The present results support the use of *in vitro* models to assess food-related factors affecting bioavailability of carotenoids and tocopherols from foods although *in vivo* and methodological factors may limit their comparability and predictive value.

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British Journal of Nutrition

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