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 threepoint (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.
by different mechanisms during digestion and absorption. In this sense, milk-derived peptides may inhibit lipid oxidation by chelation and non-chelating mechanisms\(^\text{23,24}\), show cytomodulatory and immunomodulatory effects and may bind minerals (i.e. calcium, iron, zinc)\(^\text{22,25,26}\), increasing their solubility and displaying a positive effect on mineral absorption (i.e. calcium, iron), although conflicting results have been reported\(^\text{27}\). 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\(^\text{22,28}\). 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 the effect of the presence of absorption modifiers (milk-derived peptides and iron) on the bioavailability of carotenoids and \(\alpha\)-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 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 Valles, 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 \(\alpha\)-tocopherol from fruit juices in the presence of absorption modifiers, a static gastrointestinal digestion model previously applied to fruits and vegetables was used\(^\text{29}\). 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\(^\text{30}\).

### Phytochemical analysis

For vitamins A, E (\(\alpha\)-tocopherol) and carotenoid analysis in serum, samples were processed as described elsewhere\(^\text{30}\). 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 of

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

<table>
<thead>
<tr>
<th>Type of juice</th>
<th>Lutein ((\mu)g/100 ml)</th>
<th>Zeaxanthin ((\mu)g/100 ml)</th>
<th>(\beta)-Cryptoxanthin ((\mu)g/100 ml)</th>
<th>(\beta)-Carotene (trans) ((\mu)g/100 ml)</th>
<th>(\alpha)-Tocopherol ((\mu)g/100 ml)</th>
<th>L-Ascorbic acid (mg/100 ml)†</th>
<th>Milk (%)†</th>
<th>Iron (mg/100 ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FJ</td>
<td>30</td>
<td>70</td>
<td>72</td>
<td>58</td>
<td>477</td>
<td>0.054</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>FJ+milk</td>
<td>35</td>
<td>60</td>
<td>71</td>
<td>53</td>
<td>484</td>
<td>0.054</td>
<td>11%</td>
<td>None</td>
</tr>
<tr>
<td>FJ+milk+iron</td>
<td>18</td>
<td>18</td>
<td>38</td>
<td>38</td>
<td>360</td>
<td>0.054</td>
<td>11%</td>
<td>3 mg</td>
</tr>
</tbody>
</table>

* Orange, peach, grape juice; carotenoid and \(\alpha\)-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–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 Scheffe 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.\(^{(31)}\) 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†

<table>
<thead>
<tr>
<th>Type of juice</th>
<th>Lutein (%)</th>
<th>Zeaxanthin (%)</th>
<th>β-Cryptoxanthin (%)</th>
<th>Trans-β-carotene (%)</th>
<th>α-Tocopherol (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>95 % CI</td>
<td>Mean</td>
<td>95 % CI</td>
<td>Mean</td>
</tr>
<tr>
<td>FJ§ Duodenal phase</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>11, 47</td>
<td>19</td>
<td>14, 24</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>18, 33</td>
<td>18</td>
<td>14, 22</td>
<td>30</td>
</tr>
<tr>
<td>FJ§+milk§ Duodenal phase</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>29, 36</td>
<td>31*</td>
<td>27, 35</td>
<td>51*</td>
</tr>
<tr>
<td></td>
<td>41*</td>
<td>28, 55</td>
<td>39*</td>
<td>24, 55</td>
<td>55*</td>
</tr>
<tr>
<td>FJ§+milk+iron§ Duodenal phase</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>22, 37</td>
<td>28*</td>
<td>20, 35</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>14, 44</td>
<td>25</td>
<td>6, 44</td>
<td>38</td>
</tr>
</tbody>
</table>

FJ, fruit juice. Mean values were significantly different from those of the FJ group (ANOVA and Scheffe 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 %).
Table 3. Serum levels of carotenoids and α-tocopherol at the start of each intervention period (n 32)*
(Mean values and 95 % confidence intervals)

<table>
<thead>
<tr>
<th>Type of juice</th>
<th>Lutein (μmol/l)</th>
<th>Zeaxanthin (μmol/l)</th>
<th>β-Cryptoxanthin (μmol/l)</th>
<th>Trans-β-carotene (μmol/l)</th>
<th>α-Tocopherol (μmol/l)</th>
<th>Retinol (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FJ</td>
<td>0·23 (0·19, 0·26)</td>
<td>0·04 (0·03, 0·05)</td>
<td>0·43 (0·31, 0·66)</td>
<td>0·34 (0·26, 0·43)</td>
<td>20·9 (19·6, 22·2)</td>
<td>1·64 (1·47, 1·78)</td>
</tr>
<tr>
<td>FJ + milk</td>
<td>0·25 (0·21, 0·30)</td>
<td>0·05 (0·04, 0·06)</td>
<td>0·38 (0·31, 0·43)</td>
<td>0·34 (0·26, 0·39)</td>
<td>21·5 (19·9, 23·0)</td>
<td>1·75 (1·61, 1·92)</td>
</tr>
<tr>
<td>FJ + milk + iron</td>
<td>0·26 (0·21, 0·30)</td>
<td>0·06 (0·04, 0·07)</td>
<td>0·36 (0·31, 0·40)</td>
<td>0·34 (0·26, 0·40)</td>
<td>21·8 (20·5, 23·1)</td>
<td>1·75 (1·57, 1·89)</td>
</tr>
<tr>
<td>Differences between baseline levels†</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

FJ, fruit juice.
* For details of procedures and diets, see the Subjects and methods section and Table 1.
† ANOVA and Schefte 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)

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

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.
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. 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 was 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 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, 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 and concerning the different behaviour of distinct carotenoids and tocopherols in a food under in vitro and in vivo conditions.

It has been suggested that casein phosphopeptides could inhibit lipid oxidation by chelation and non-chelating mechanisms such as free radical scavenging. 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 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. 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. 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, although comparisons are often difficult since both approaches lack standardization parameters or traceability to reference methods. 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 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.

Acknowledgements

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References


