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Carotenoids bioaccessibility in pulp and fresh juice from carotenoid-rich sweet oranges and mandarins

María Jesús Rodrigo[a*], Antonio Cilla[b], Reyes Barberá[b], Lorenzo Zacarías[a]

[a]Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (IATA-CSIC), Avda. Agustín Escardino 7, 46980 - Paterna (Valencia), Spain.

[b]Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Avda. Vicente Andrés Estellés s/n, 46100 - Burjassot (Valencia), Spain.

* Corresponding author at Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (IATA-CSIC), Avda. Agustín Escardino 7, 46980 - Paterna (Valencia), Spain. Telephone: +34-96 3900022; fax +34-96 3636301.

Email address: mjrodrigo@iata.csic.es (M.J. Rodrigo)

Abstract

Citrus fruits are a good source of carotenoids for the human diet; however, comparative studies of carotenoids in different citrus food matrices are scarce. In this work the concentration and bioaccessibility of carotenoids in sweet oranges and mandarins with marked differences in carotenoid composition were evaluated in pulp and compared to those in fresh juice. The pulp and juice of the red-fleshed Cara Cara sweet orange variety was highly rich in carotenes (mainly lycopene and phytoene) compared to standard Navel orange, while β-cryptoxanthin and phytoene predominated in mandarins. Total carotenoid content in the pulp of the ordinary Navel and in the red-fleshed Cara Cara orange, as well as and in Clementine mandarin were higher than in the corresponding juices, although individual carotenoids were differentially affected by juice preparation. Bioaccessibility of the bioactive carotenoids (the ones described to be absorbed by humans) was greater in both pulp and juice of the carotenoid-rich Cara Cara orange compared to Navel while increasing levels of β-cryptoxanthin were detected in the bioaccessible fractions of pulp and juice of mandarins postharvest stored at 12ºC compared to freshly-harvested fruits. Overall, results indicated that higher soluble bioactive carotenoids from citrus fruits and, consequently, potential nutritional and health benefits are obtained by the consumption of pulp with respect to fresh juice.

Keywords: Bioaccessibility; Carotenoids; Citrus fruits; *in vitro* digestion; Juice; Pulp

Abbreviations:

BF: bioaccessible fraction

CC: Cara Cara orange

M: Clementine mandarin

M12: Clementine mandarin stored at 12 ºC for 5 weeks

MTBE: methyl tert-butyl ether

N: Washington Navel orange

Introduction

Fresh citrus fruits and their juices are recognized as rich sources of vitamins and other important bioactive compounds with diverse biological activities.[1] Among relevant phytochemicals present in the edible portion of citrus fruits, carotenoids - a large family of isoprenoid compounds - are of special relevance due to their dual effect on human nutrition and health-related benefits. First, specific carotenoids, such as β–, α-carotene and β-cryptoxanthin, are precursors of vitamin A and accumulate in citrus fruits, being a good source to fulfill the recommended daily ingestion specifications.[2] On the other hand, the regular intake of certain carotenoids, such as β-cryptoxanthin or lycopene, contained in significant proportions in fruits of specific citrus species and varieties, has been correlated with a reduced risk of developing certain chronic diseases and cancers, improved bone health, or reduction in obesity.[3,4,5] In addition, carotenoids are the pigments responsible for the attractive color of citrus fruits and, therefore, have a strong influence on fruit external and internal appearance, its marketability and consumer acceptance.

The carotenoid profile in the pulp tissue or juice vesicles, has been described for different citrus species, including many varieties of sweet orange and mandarin.[6,7,8] The predominant carotenoids in the pulp of ordinary sweet orange and mandarin are β,β-xanthophylls, being the *9-Z* isomer of violaxanthin the main carotenoid in sweet orange and β-cryptoxanthin in mandarins, while other xanthophylls such as antheraxanthin, zeaxanthin, lutein, and colorless carotenes are also present in lower proportions.[7,9,10] The qualitative composition of carotenoids in processed citrus juice is similar to that described for pulp. However, a more complex pattern of carotenoids is generally found in juice, due to the formation of isomers and rearrangements of epoxy groups under the acidic juice condition and the thermal or other stabilization treatments applied during juice processing.[11,12,13]

Among the carotenoids found in citrus fruit, lycopene is very unusual and only three varieties of sweet orange have been described to accumulate lycopene.[14] CaraCara (CC) sweet orange is a spontaneous bud mutation from the Washington Navel (N) orange, with an attractive bright red pulp color due to lycopene accumulation in the juice vesicles (Fig. 1), while other ripening parameters including external fruit coloration are similar to parental fruits. In addition to lycopene, the pulp of CC accumulates exceptionally high amounts of phytoene and phytofluene,[14] two carotenes with potential health and nutritional benefits.[15,16] Therefore, the CC orange has an exceptional high carotenoid content compared to other sweet oranges, and may be particularly interesting for nutritional or functional studies of uncommon carotenes in a citrus food matrix.

Citrus fruits are one of the main dietary sources of β-cryptoxanthin, and in particular, the mandarin fruits contain the highest concentrations of β-cryptoxanthin within the genus *Citrus*.[9,17,18] Moreover, it has recently been reported that postharvest storage of citrus fruit at moderate cold temperature (12ºC) enhanced carotenoid accumulation and, specifically, the concentration of β-cryptoxanthin in pulp.[19] This suggests that storage at 12ºC is a feasible postharvest strategy for improving the nutritional and functional value of citrus fruits.

The health-related effects of carotenoids depend not only on the amount consumed but also on their bioavailability. Carotenoid bioavailability in humans is usually assessed by monitoring changes in plasma carotenoid concentrations after the ingestion of carotenoid-rich foods. These studies are expensive and lengthy, however studies involving animal models have limitations, since the carotenoid bioavailability, metabolism and utilization parameters in such situations differ from those found in humans.[20] Simulated *in vitro* digestion allow to estimate bioaccessibility, i.e., the amount of a food component released from the food matrix and which constitutes the maximum amount available for absorption. Bioaccessibility can be used to evaluate the relative bioavailability of carotenoids.[21] A limited number of studies have addressed the characterization of carotenoid bioaccessibility from the whole intact pulp of an orange fruits,[2,10,20,21] and most of them have focused on their juice (hand-squeezed orange and mandarin juices);[10,22] milk-based fruit drink (containing 48% mandarin juice);[23] hand-squeezed orange juice;[24] milk and soy-based fruit beverages (containing orange);[25] fresh industrial-squeezed orange juice;[10,26] and blended fruit juice (containing 50% orange).[27]

Up to date only one study in citrus fruits has compared the bioaccessibility of carotenoids between fruit segments (with or without homogenization) and juice (fresh or pasteurized).[10] Results pointed out that *in vitro* bioaccessibility of main bioactive carotenoids (those that are regularly detected in human plasma)[28], as zeaxanthin, α- and β-carotene, lutein and β-cryptoxanthin was improved in juice compared to fruit segments most likely due to the presence of fibrous matrix compounds in segment products.[10] However, bioaccessibility of the carotenes phytoene and phytofluene that may exert potential health benefits, has been relatively unexplored in citrus food matrices despite these carotenes can be at medium or high level in widely consumed citrus fruits like oranges, grapefruits or mandarins.[15] In this context, expanding this kind of studies to other citrus fruit varieties or species with a rich composition in specific carotenoids would provide valuable information to better understand carotenoid distribution and bioaccessibility in different matrices. Therefore, the main objective of the present work has been to investigate the effect of citrus food matrix (pulp of the fruit *versus* fresh juice) on individual carotenoid content and their content in bioaccessible fraction and relative bioaccessibility of bioactive carotenoids in two sweet oranges with marked differences in carotenoid composition: the standard Navel and red-fleshed pulp CaraCara which is highly rich in carotenes (phytoene, phytofluene and lycopene), and in freshly harvested and refrigerated Clementine mandarins with elevated content of the provitamin A carotenoid β-cryptoxanthin.

Materials and methods

Reagents

Carotenoid analysis: HPLC-grade methanol, chloroform and acetone were supplied by Scharlau (Barcelona, Spain) and methyl tert-butyl ether (MTBE) by Merck (Darmstadt, Germany). Petroleum ether and diethyl ether were of analytical grade and were supplied by Scharlau (Barcelona, Spain). Commercial standards of β-carotene (≥97%), lutein (≥95%)and lycopene (≥90%)were purchased from Sigma-Aldrich, and β-cryptoxanthin (≥97%) and zeaxanthin (≥98%) from Extrasynthese (Lyon, France). Standards of phytoene and phytofluene were obtained from peel extracts of Pinalate orange fruits, and of all-*E*-violaxanthin and 9-*Z*-violaxanthin from peel extracts of Navel orange fruits and HPLC purified.[29,30]

Simulated gastrointestinal digestion: Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis, MO, USA): pepsin (porcine, 975 units/mg protein), pancreatin (porcine, activity equivalent to 4 x USP specifications) and bile extract (porcine). Working solutions of these enzymes were prepared immediately before use.

Samples

Mature fruits of Washington Navel and the red-fleshed mutant CaraCara sweet oranges (*C. sinensis* [L.] Osbeck) at full mature stage (soluble solid content 13 ºBr and maturity index of 8.3 for Navel and a soluble solid content of 12 ºBr and maturity index of 7.9 for CC) were harvested in December 2010, from adult trees from the Citrus Germplasm Bank at the Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada, Valencia, Spain). Clementine mandarins (*Citrus clementina*) (M) were also harvested at full mature stage in December 2010 (soluble solid content 12 ºBr and a maturity index of 12.2) from a commercial orchard located in Lliria (Valencia, Spain). Immediately after harvesting fruits were delivered to the laboratory, divided into two lots of at least 70 fruits each. One lot of fruits was used to obtain the pulp tissue by slicing the fruits in half and then excising small cube pieces of approximately 1 cm^2 of fruit segments (juice vesicles), immediately frozen in liquid nitrogen and stored at -80°C until analysis. The second lot was used to obtain the fruit juice with a household electric hand reamer (Citromatic MPZ22, Braun, Barcelona, Spain), that was filtered through a metal sieve with a pore size of 0.8 mm, immediately frozen in liquid nitrogen and stored at -80ºC until analysis. Two additional lots of Clementine mandarins were stored in postharvest room chambers at 12ºC and 90-95% relative humidity for 5 weeks (M12). After that period, fruits were processed as before to obtain the intact pulp and juice samples. This storage postharvest condition was used to stimulate carotenoids content in the pulp of mandarin fruit as previously reported in other citrus fruits.[19]

In vitro digestion

An *in vitro* gastrointestinal digestion procedure mimicking the physiological conditions in the upper digestive tract (stomach and small intestine) was used to evaluate the bioaccessibility of carotenoids according to Cilla *et al.* [25]

Briefly, 80 g of citrus fruit pulps or juices were adjusted to pH 2.0 with 6 M HCl (GLP 21 pH-meter, Crison, Barcelona, Spain). The pH was checked after 15 min, and if necessary readjusted to 2.0. Then an amount of freshly prepared demineralized pepsin solution sufficient to yield 0.02 g pepsin/g sample was added. The samples were made up to 100 g with cell culture-grade water (Aqua B Braun, Braun Medical, Barcelona, Spain), and incubated in a shaking water bath at 37ºC/120 strokes per minute for 2 h (SS40-2, Gran Instruments, Cambridge, UK). The gastric digests were maintained in ice for 10 min to stop pepsin digestion. For the intestinal digestion stage, the pH of the gastric digests was raised to pH 6.5 by the dropwise addition of 1 M $NaHCO_3$. Then an amount of freshly prepared and previously demineralized pancreatin-bile salt solution sufficient to provide 0.005 g pancreatin and 0.03 g bile salt/g sample was added, and incubation was continued for an additional 2 h. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. The pH was then adjusted to 7.2 by the dropwise addition of 0.5 M NaOH.

To quantify the amount of carotenoids transferred to the aqueous-micellar fractions, aliquots of 25 g of sample were transferred to polypropylene centrifuge tubes (50 ml, Costar, New York, USA) and centrifuged at 3500 x g for 1 h at 4ºC (GT422 centrifuge, Jouan, Saint Nazaire, France). Supernatants (aqueous-micellar fraction considered the bioaccessible fraction, BF) obtained after *in vitro* digestion were immediately frozen at -80ºC and used to determine carotenoid contents. Relative bioaccessibility (%) refers to the amount of tested compound remaining in the bioaccessible fraction related to the original non-digested sample. This parameter can be calculated as follows: 100 x (bioaccessible content/total content).

Determination of carotenoids

Carotenoids were extracted and analyzed as described by Alquezar *et al.* [14] and Rodrigo *et al.*[29], with some minor modifications. Frozen pulp sections were ground to a fine powder under liquid nitrogen using an electric mill grinder (Taurus, Barcelona, Spain) prior analysis. Briefly, freeze-ground pulp (5 g) was weighted in screw-capped polypropylene tubes (50 ml), and 8 ml of MeOH were added. Juice (15 mL) or bioaccessible fraction (25 mL) were defrosted at room temperature and 8 ml of MeOH were added. The suspension was stirred for 10 min at 4 ºC. Tris-HCl (50 mM, pH 7.5) (containing 1 M NaCl) was then added (5 mL) and further stirred for 10 min at 4ºC. Chloroform (10 mL) was added to the mixture, stirred for 5 min at 4 ºC and centrifuged at 3000 g for 10 min at 4ºC. The hypo-phase was removed and the aqueous phase re-extracted with chloroform until it was colorless. The pooled chloroform extracts were dried on a rotary evaporator at 40ºC and were saponified in methanolic solution of KOH (6% w/v) overnight at room temperature. Saponified carotenoids were recovered from the upper phase after adding 5 ml of MilliQ water and 10 ml of solution A (petroleum ether:diethyl ether, 9:1) to the mixture. Repeated re-extractions by adding 5 ml of solution A were carried out until the hypo-phase was colorless. The extracts were reduced to dryness by rotary evaporation at 40ºC and quantitatively transferred to a Pyrex tube with chloroform and acetone. In order to precipitate the sterols present in the samples, the acetone extracts were kept overnight at –20 ºC and centrifuged at 3000 g for 5 min at 4 ºC. The supernatant was transferred to a 1.5 mL vial, dried under N_2 and kept at -20 ºC until HPLC analysis. All operations were carried out on ice under dim light to prevent photodegradation, isomerization and structural changes of the carotenoids. At least four replicates of each sample were analyzed.

The carotenoid composition of each sample was analyzed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a model 2998 photodiode array detector, and Empower software (Waters, Barcelona, Spain). A C30 carotenoid column (250 x 4.6 mm, 5 μm) coupled to a C30 guard column (20 x 4.0 mm, 5 μm) (YMC, Teknokroma, Spain) was used. Samples were prepared for HPLC by dissolving the dried carotenoid extracts in $CHCl_3$: MeOH: acetone (3:2:1, v:v:v). Ternary gradient elution was used for carotenoid separation. The initial solvent composition consisted of 90% MeOH, 5% water and 5% MTBE. The solvent composition changed in linear fashion to 95% MeOH and 5% MTBE at 12 min. During the next 8 min the solvent composition was changed to 86% MeOH and 14% MTBE. After reaching this concentration, the solvent was gradually changed to 75% MeOH and 25% MTBE at 30 min. After 20 min, the solvent composition changed linearly, being 50% MeOH and 50% MTBE at 50 min. The final composition was reached at 70 min, and consisted of 25% MeOH and 75% MTBE. The initial conditions were re-established in 5 min and equilibrated for 15 min before the next injection. The flow rate was 1 mL/min, column temperature was set to 25°C, and the injection volume was 20 μL. The photodiode array detector was set to scan from 250 to 540 nm, and for each elution a Maxplot chromatogram was obtained, plotting each carotenoid peak at its corresponding maximum absorbance wavelength.

Carotenoids were identified by their retention time, absorption and fine spectra.[28,30,31] The carotenoid peaks were integrated at their individual maximal wavelength, and their contents were calculated using the appropriate calibration curves, as described elsewhere.[14,19]

Statistical analysis

The results shown represent mean values ± standard deviation, and were calculated from the means of four replicates obtained in at least two separate experiments. One-way (type of sample) ANOVA was conducted, followed by Tamhane´s T2 multiple comparison test, since the variances were unequal. The level of significance was set at $p < 0.05$ (SPSS version 17.0 statistical package, Chicago, IL, USA).

Results and discussion

Carotenoid profiling and content in the pulp of citrus fruit *versus* juice

A comparative study was made to assess whether the citrus fruit matrix, juice or pulp, affected the total and individual carotenoid contents in two different species of citrus fruits with distinctive carotenoid content and composition. On one hand, we selected and compared the ordinary sweet orange Washington Navel (*Citrus sinensis*) and its spontaneous red-fleshed mutant Cara Cara, which contains large proportions of linear carotenes, highlighting the presence of lycopene in the pulp.[14] As shown in Fig. 1, the pulp color of CC oranges was clearly distinguishable from the parental Navel, due to the red pigmentation as compared with the orange-yellowish color of the Navel orange. Furthermore, the fresh juice from CC orange also showed a more intense dark-orange tint than the Navel variety (Fig. 1). It is interesting to note that after juice preparation, the juice vesicle membranes retained in the filter sieve showed a pale pink color in the case of CC, but were colorless or pale yellow in the case of Navel. A second citrus species, the Clementine mandarin (M), was selected for this study by the high quality of the fruit and the elevated proportion of β-cryptoxanthin in the pulp compared to sweet orange.[18] In M the carotenoid composition in pulp and hand-squeezed juice from freshly harvested fruits was compared to that of fruit stored for 5 weeks at 12ºC (M12). The purpose of this postharvest treatment was to enhance the accumulation of carotenoids in the pulp of mandarins, in particular upstream carotenes and β,β-xanthophylls as reported previously for other citrus fruit.[19] The pulp and juice of freshly harvested M fruits showed an intense orange color that was visually appreciated as darker orange in fruits stored at 12ºC (Fig. 1). The juice vesicle membranes retained in the filter sieve after juice preparation of both freshly and refrigerated stored M fruits showed a pale orange coloration.

Twenty-five different carotenoid-like peaks were separated in our chromatographic conditions from pulp and juice extracts of sweet oranges and mandarins (Table 1), in good agreement with the complex carotenoid pattern described for citrus juices.[10,12] Nine carotenoids (*15-Z*-phytoene, phytofluene, ζ-carotene, *all-E*-lycopene, *all-E*- and *9-Z*-violaxanthin, zeaxanthin, β-cryptoxanthin and β-carotene) were unambiguously identified by comparing chromatographic and spectroscopic characteristics with standards, while *Z*-antheraxanthin was tentatively identified. The remaining peaks showed the characteristic carotenoid absorption spectrum[32] but were not ascribed to a specific carotenoid. Lycopene and an additional isomer of phytofluene were only identified in CC extracts while peaks 13 to 15, showing absorption spectrum and retention time similar to different *Z*-isomers of β-cryptoxanthin[28] were only detected in mandarin samples. Since chromatogram peak area of the nine identified carotenoids plus antheraxanthin comprised the main bioactive carotenoids and accounted for more than 95 % of the total area in the chromatograms, only these carotenoids were considered in this study.

Total carotenoid content, as the sum of individual carotenoids, was determined in pulp and juice of the selected sweet orange and mandarin fruits. In all varieties, total carotenoid content was lower in juice compared to pulp, though differences were observed among samples (Tables 2 and 3). In Navel oranges, the carotenoid content in pulp was approximately 9700 ng/g fresh weight, while in the fresh juice the content decreased by half (Table 2). The total carotenoid content in Navel juice is lower than hand-squeezed juices from Valencia late oranges,[24] but similar to that of other Navel oranges (reviewed in Alquezar *et al.*[7]). It is known that carotenoid content is highly variable depending on the citrus fruit variety. Therefore, the lower carotenoid content in Navel pulp and juice may be an intrinsic characteristic of this variety. Carotenoid content in CC pulp was 10-times higher than in the parental Navel, in agreement with previous data.[6,14,33] The proportion of carotenoids in CC juice was one-fifth of that in pulp (Table 2), indicating greater carotenoid losses during juice extraction compared to that of Navel orange. Nevertheless, the total carotenoid content in CC juice was almost 5-times higher than in Navel juice, thus pointing to this variety as an exceptionally carotenoid-rich citrus fruit in both pulp and juice matrices.

Postharvest storage of citrus fruit at moderate temperature (8-15ºC) has been shown to stimulate carotenoid biosynthesis in both pulp and peel tissues, increasing fruit coloration without any detrimental effect on sensorial quality.[19,34] Storage of M for 5 weeks at 12ºC promoted accumulation of carotenoids in the pulp, increasing the total content by 50% compared with that of freshly harvested fruits (Table 3). These results are consistent with those obtained with sweet oranges, where a two-fold increase in total carotenoid content in the pulp after 7 weeks of storage at 12ºC was observed.[19] Similarly to Navel oranges, a reduction of approximately 40% in total carotenoid content was detected in M juice compared to pulp in both freshly harvested and stored fruits (Table 3). This general decrease in total carotenoid content in M juice compared to pulp suggests that a significant proportion of carotenoids is retained in the juice vesicle membranes discarded during the filtering process. Therefore, considering the losses in total carotenoid concentration observed in the samples of oranges and mandarins during juice processing, the intake of intact citrus pulp or the juice supplemented with juice vesicle membranes would be more recommended. However, a recent study indicates that the presence of pectin and dietary fiber in sweet orange derived products may adversely affect carotenoids bioaccessibility.[10]

In order to determine whether the decrease in total carotenoid content observed in juice compared to pulp affects all carotenoids or it is restricted to only some of them, we determined the individual carotenoid compositions in pulp and juice samples from sweet oranges and mandarins. Seven major carotenoids were identified in the pulp and juice from Navel oranges: the colorless phytoene and phytofluene, and the β,β−xanthophylls β-cryptoxanthin, zeaxanthin, antheranxanthin (*Z*-isomer), and violaxanthin (*all-E* and *9-Z* isomers) (Table 2). As expected, more than 90% of the total carotenoid content corresponded to β,β,-xanthophylls, being *9-Z*-violaxanthin the predominant carotenoid (Table 2 and Fig.1S ESI). The percentage of each individual carotenoid with respect to the total was similar in pulp and juice (Fig. 1S ESI) of Navel fruit, although a slight reduction in the proportion of antheraxanthin and an increase in β-cryptoxanthin were observed in the juice compared to pulp. In CC pulp and juice, we detected lycopene and β-carotene, and an additional isomer of phytofluene, in addition to the carotenoids identified in Navel oranges (Table 2). The most remarkable features of CC samples were the presence of lycopene, which was totally absent in ordinary sweet oranges, and the elevated concentrations of phytoene and phytofluene, which were between 25- and 130-times higher, respectively, than in Navel (Table 2). Overall, linear carotenes in CC samples accounted for 94% and 80% of the total carotenoid content in pulp and juice, respectively, while in Navel these carotenes only accounted 5 and 7% of the total, respectively (Fig. 1S, ESI). Moreover, the concentration of β,β-xanthophylls in CC pulp and juice was reduced approximately 45% compared with the

Navel samples (Table 2). These results are in agreement with previous data, and suggest a metabolic alteration in the carotenoid pathway in CC fruits which may involve an increased flux of substrates into the pathway combined with partial blockage downstream of lycopene cyclization.[14] Due to the significant amounts of lycopene detected in CC samples, especially in whole intact pulp, and the health benefits associated to the intake of this carotene,[4] CC oranges provide an added value to consumers compared to ordinary sweet oranges, although the potential bioaccessibility and bioavailability of lycopene in this fruit (pulp or juice) remains to be evaluated.

As in Navel oranges, the concentration of the main β,β-xanthophyll, *9Z*-violaxanthin, was reduced approximately by half in CC juice compared with the pulp (Table 2). However, in CC juice the content of linear carotenes was more extensively reduced, and the concentration of phytoene and lycopene decreased 5-times, and 20-times that of phytofluene (Table 2). These results suggest that xanthophylls may accumulate preferentially in the cells of the central cavity of the orange juice vesicles or/and are more easily transferred from the vesicle epidermal cells to the juice during the extraction process, while carotenes may be associated to the epidermal cells and are highly retained in this fraction. In this sense, it is known that xanthophylls and lycopene accumulate in different subcellular structures, since xanthophylls are usually stored in plastoglobuli and lycopene accumulates in crystalloid substructures.[35,36] Therefore, the differential carotenoid accumulation structure may have a crucial effect on the stability of these compounds in the juice and/or their release from the vesicle cells into the juice fraction.

In the pulp of M12 an increase of approximately 40% in total carotenoid content was observed compared with M fruits (Table 3), in agreement with previous results showing an enhancement of internal and external color and carotenoid content in citrus fruits stored between 10-15ºC.[19] Total carotenoid content in the pulp, 9424 and 13503 ng/g fresh weight in M and M12, respectively, were slightly lower to those reported for fruits of other Clementine mandarins.[18] However, the influence of the cultivar, climatic conditions and agronomical practices are likely to be responsible for these quantitative differences. As in ordinary sweet orange, the proportion of β,β-xanthophylls represented about 90% of the total carotenoid content in the pulp of both M and M12 fruits, being β-cryptoxanthin was the most abundant, accounting for 40% and 48% of the total in M and M12, respectively (Table 3 and Fig. 2S ESI), followed by violaxanthin (sum of both *all-E* and *9-Z* isomers), which was about 30% of the total, and zeaxanthin and antheraxanthin as minor components (Table 3 and Fig. 2S, ESI). It is interesting to note that storage at 12ºC specifically induced accumulation of β-carotene and β-cryptoxanthin, increasing then the total provitamin A activity of the pulp.

In accordance with the results obtained in ordinary sweet orange, total carotenoid content in the juice of M and M12 fruit was reduced by 40% compared with the content of the pulp, though not all carotenoids were similarly affected (Table 3). The concentrations of the carotenes phytoene and β–carotene in the juice of M and M12 samples were similar or slightly greater than in the pulp but the level of β-cryptoxanthin in the juices was around 80% of that in pulp. However, other β,β-xanthophylls were more severely affected, and their contents were reduced to values ranging from 14% to 27% of those in pulp (Table 3).

Carotenoid bioaccessibility

Total and individual carotenoid content in the bioaccessible fractions of the samples of orange and mandarin analyzed are shown in Tables 2 and 3, respectively. It is noticeable that the pulp of CC oranges exhibited a total carotenoid content in the bioaccessible fraction (BF) over 10-times higher than that of N oranges. Furthermore, the individual carotenoid profile differed between both oranges and a larger number of carotenoids were identified in the BF of CC variety, mainly due to the presence of phytoene, phytofluene, lycopene and β-carotene (Table 2). It should be noted that phytoene (3308 ng/g) and phytofluene (454 ng/g) predominated in the BF of CC pulp. This is consistent with the greater presence of these carotenes in pulp before digestion. As for xanthophylls, the concentration in the BF was of the same order in both varieties, despite the greater content observed in the pulp of N versus CC before digestion (Table 2).

Regarding the juices, total carotenoid content in the BF were approximately 44-times higher in CC versus N. In the CC variety, the BF of the juice presented greater contents of carotenes and xanthophylls compared with N oranges, particularly phytoene and phytofluene (Table 2). Although in both varieties the total carotenoid content in the BF was greater in pulp than in juice, variations were observed depending on the variety and carotenoid considered.

M12 exhibited a content of total and individual carotenoid in the BF of the pulp two-times higher than in M. It is also remarkable the presence of large concentrations of β-cryptoxanthin -the main carotenoid with provitamin A activity in mandarins- in the BF of M and M12, representing 54% and 59% of total carotenoids in the BF, respectively. The carotene content in the BF of the juice did not show marked differences between M and M12. Nevertheless, in the case of the xanthophylls, the content was greater in M12 than in M, justifying the greater total carotenoid content in M12 versus M juices (Table 3). Likewise, for all carotenoids analyzed in the BF of mandarins, a higher content was observed in pulp than in juice, independently of the type of sample analyzed (M or M12).

The comparison of carotenoid bioaccessibility data is difficult due to differences in the *in vitro* digestion model used as well as in the citrus variety analyzed. Concerning the *in vitro* digestion models applied to obtain the micellar fraction for the determination of carotenoids bioaccessibility [used as an estimation of the relative bioavailability of carotenoids (bioaccessibility) and potentially available carotenoids], distinct procedures have been applied. Three different protocols have been evaluated, comprising overnight decantation, low-speed centrifugation (5000 rpm/20 min) and ultracentrifugation (12900 rpm/2 h and 25000 rpm/30 min).[2,21] According to these authors, the best recovery and the more practical conditions were obtained with low-speed centrifugation. Therefore, different authors measuring citrus carotenoids bioaccessibility have applied low-centrifugation step[24,27] as in the present study but others used ultracentrifugation with[10,22,37] or without[20] filtration.

Different studies indicated that xanthophyll carotenoids are more bioaccessible than carotenes because are more efficiently transferred to the micelles.[2,10,20,21,27] This has been observed in both pulp and juice of Navel oranges and M and M12 mandarins. However, in the case of the CC variety, carotenes were more bioaccessible than xanthophylls due to the high total and bioaccessible content of phytoene and phytofluene in pulp and juice. It should be mentioned the absence of results in literature of the bioaccessibility of phytoene and phytofluene from oranges or mandarins in both matrix, pulp or juice. The presence of the colorless carotenes phytoene and phytofluene has been reported in numerous carotenoid-containing fruits and vegetables,[16] however, information on their bioaccessibility and bioavailability is mainly restricted to tomato as a whole fruit or derived products.[15,16,38,39,40] The study performed on the bioaccessible content of phytoene in red grapefruit[36] is in good agreement with our results and pointed to phytoene as the main carotenoid in the BF compared with the rest of carotenoids (α- and β- carotene, lycopene, lutein and violaxanthin).

The relative bioaccessibility of the carotenoids identified in the pulp or juice, that has been previously detected in the human plasma[28], are represented in Figs. 2 and 3, for oranges or mandarins, respectively. Xanthophyll epoxides, which are not found in human plasma and tissues, are considered not be absorbed by humans, and their bioaccessibility is not relevant and has not been calculated in this study.[10,24,41] Carotenoid relative bioaccessibility from pulp of the CC variety followed the order: β-cryptoxanthin (11%) > β-carotene (5.8%) > phytoene = phytofluene (4%) > lycopene 0.8%, while in the case of the N variety only phytoene (8.5%) and β-cryptoxanthin (6.6%) were bioaccessible among the considered bioactive carotenoids (Fig. 2). The comparison of these data with other studies, as aforementioned, is difficult due to differences in the *in vitro* digestion model used as well as in the orange variety analyzed. Accordingly, different trends in the bioaccessibility among carotenoids analyzed in each study were found. Higher bioaccessibilities have been reported for β-cryptoxanthin (97.8%), zeaxanthin (102.8%), lutein (102.5%) and β-carotene (33.6%) in the edible portions of oranges.[20] Similarly, higher bioaccessibility of β-cryptoxanthin (34.5, 37.3%) versus β-carotene (3.6, 6.6%) has been reported for orange segments and homogenates, respectively.[10] However, other study with orange fruit showed the highest bioaccessibility for β-carotene (55%), followed by β-cryptoxanthin (41.3%), zeaxanthin (38.9%) and lutein (25.8%).[21] In a mixture of fruits containing 47% oranges and 12% mandarins, the bioaccessibility of β-carotene (13.8%) was found to be greater than that of lutein (8.3%) and lycopene (1.5%).[42] Interestingly, in the only study in which the phytoene bioaccessibility was determined in a citrus fruit (red grapefruit), this carotene showed the highest bioaccessibility (47%), followed by lutein (8.7%), violaxanthin (8.4%) and β-carotene (7.9%), while the lowest percentage corresponded to lycopene (4.5%).[37] The high relative bioaccessibility of phytoene is important since this carotene absorbs UV light and offers better protection than other carotenoids against skin exposure to UV radiation and the potential harmful effects.[15,37,16]

Carotenoid relative bioaccessibility was greater in N and CC juices than in the corresponding pulp samples in agreement with a previous study showing a 2.6-fold higher carotenoid bioaccessibility of orange juices compared to orange segments.[10] In N juice, only phytoene (22.6%) and β-cryptoxanthin (3%) were detected in the bioaccessible fraction. In CC juice, relative bioaccessibility followed the order: phytofluene (82%) > β-carotene (22%) > phytoene (19.5%) > β-cryptoxanthin (16%) > lycopene (2%). Interestingly, the bioaccessibility of phytofluene has only been assessed to date in tomato extracts or derived products, since these are the most prominent source of this carotene in a Western diet.[15,16] Taking this into consideration, CC oranges, in particular CC juice, is an excellent source for investigating the bioactivity of this carotene, due to its high relative bioaccessibility (Fig. 2).

In mandarins (Fig. 3), the pulp showed greater relative bioaccessibility than the corresponding juices, with the exception of zeaxanthin in M12. The pulp of M12 showed greater relative bioaccessibility (27-30%) than the pulp of M (14-20%) for all the carotenoids studied. The relative bioaccessibility of phytoene was similar (13%) in the juices of M and M12, while the relative bioaccessibilities of β-carotene, β-cryptoxanthin and zeaxanthin were higher in M12. The relative bioaccessibility of the latter was found to be particularly high (67%). Elevated bioaccessibility of xanthophylls can be due to esterification, since it has been reported that the bioaccessibility of β-cryptoxanthin seems to be inversely related to the degree of esterification.[22] However, in the present work we did not consider the grade of esterification of the xanthophylls zeaxanthin and β-cryptoxanthin, since it has been described that esters are readily cleaved to free xanthophylls during the duodenal stage of digestion.[35]

The bioaccessibility of carotenoids with provitamin A activity (β-cryptoxanthin and β-carotene) has been investigated in hand-squeezed juices of sweet orange and mandarin.[22] The bioaccessibility of β-cryptoxanthin and β-carotene in mandarin juices were between 16-18% and 26-31%, respectively, versus 22% and 33% in the case of orange juice. These values are similar to those obtained in our study for β-cryptoxanthin and β-carotene in CC juice (16.1% and 22.38%, respectively) or M12 juice (20.25% and 18%). Industrial extraction of orange juice increases carotenoid bioaccessibility versus hand-squeezing, since industrial extraction reduces the pulp particle size and enhances carotenoid bioaccessibility. The relative bioaccessibilities of the bioactive carotenoids from industrially-squeezed versus hand-squeezed fruit are approximately: β-carotene 50% versus 30%, α-carotene 50% versus 40%, β-cryptoxanthin 55% versus 35%, zeaxanthin 50% versus 30% and lutein 50% versus 30%.[24] Other study with fresh industrially-squeezed orange juices found the relative bioaccessibility of the bioactive carotenoids (β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin and lutein) to be between 40-50%.[26] A more recent study with freshly squeezed orange juice reported the following order of carotenoids bioaccesibility: β-cryptoxanthin 55.9% > zeaxanthin + (9Z)-antheraxanthin 30% > lutein 23.9% > β-carotene 7.7% > α-carotene 4.4%. [10]

Conclusions

In this study we investigated the carotenoid contents and their content in bioaccessible fraction and relative bioaccessibility of bioactive carotenoids of different varieties of two citrus species highly consumed worldwide, sweet orange and mandarins. Moreover, the selection of citrus varieties highly rich in carotenoids, i.e., CC sweet orange with unusual lycopene and colorless carotene accumulation, and Clementine M12 mandarins (postharvest stored at 12 ºC) with elevated provitamin A β-cryptoxanthin content, allowed investigation of the distribution of specific bioactive carotenoids in both citrus food matrices. In general, the qualitative carotenoid composition was the same in pulp and juice for a given variety, although a reduction of approximately 40% in total carotenoid content was observed in freshly prepared juice compared to pulp. However, this effect was not equal for all carotenoids, and clearly depended on the citrus variety thus underscoring the need to evaluate individual carotenoid losses during juice preparation for each citrus species or variety. Interestingly, both CC pulp and juice accumulated high levels of phytoene, phytofluene and lycopene compared with the parental Navel orange, whereas the M12 pulp and juice were rich in β-cryptoxanthin compared with control mandarins.

Taking together the results of carotenoid relative bioaccessibility and considering the functionality of bioactive carotenoids (phytoene, phytofluene, lycopene, β-carotene, β-cryptoxanthin and zeaxanthin), pulp and juice derived from CC orange appears to be a more convenient option, than those from Navel variety. In addition, the bioaccessible content (amount available for absorption) of bioactive carotenoids in CC orange pulp and juice were similar. On the other hand, in the case of mandarins, postharvest storage at 12 ºC increases bioactive carotenoids, specifically β-cryptoxanthin content, in pulp and juice bioaccessible fractions, compared with freshly harvested mandarins. In summary, the pulp of citrus fruits contains similar or higher content of soluble bioactive carotenoids respect to fresh juice and, consequently, increased potential nutritional and health benefits may be acquired by consumption of this food matrix.

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Fig. legends

Fig. 1

Appearance of pulp and freshly hand-squeezed juice of the sweet orange Washington Navel (N), the red-fleshed Cara Cara (CC) orange, Clementine mandarins (M) and Clementine mandarins stored for 5 weeks at 12 ºC (M12).

Fig. 2

Relative bioaccessibility (%) of the bioactive carotenoids in pulp and freshly hand-squeezed juice of the sweet orange Washington Navel (N) and the red-fleshed Cara Cara (CC) orange. Data are mean ± SD (n=4). Different letters represent significant differences ($p<0.05$) for a carotenoid among samples.

Fig. 3

Relative bioaccessibility (%) of the bioactive carotenoids in pulp and freshly hand-squeezed juice of Clementine mandarins (M) and Clementine mandarins stored for 5 weeks at 12 ºC (M12). Data are mean ± SD (n=4). Different letters represent significant differences ($p<0.05$) for a carotenoid among samples.

Table 1 Chromatographic and spectroscopic characteristics of carotenoids found in sweet orange and mandarin pulp and juice samples

Peak	Retention time	Carotenoid[a]	UV-Vis absorption maxima (nm)	D_B/D_{II}[b]
1	14.0	NI	327(Z), 405, 429, 456	0.21
2	14.9	NI	327(Z), sh, 430, 460	0.19
3	15.7	**All- E*-violaxanthin	415,438,468	0.07
4	17.9	NI	397,420,448	
5	18.9	Mix NI	328(Z), 399,323,438,446,462	0.45
6	21.0	**9-Z*-violaxanthin	328(Z),412,438,465	0.08
7	22.2	NI	390,416,442	
8	23.2	NI	sh, 427,451	
9	24.0	NI	sh,442,470	
10	25.5	NI	418,444,473	
11	26.5	*Zeaxanthin	430,450,478	
12	27.2	Z-Antheraxanthin	329(Z), 419,441,468	0.07
13	28.4	NI-M	328(Z),sh,444,472	0.06
14	29.0	NI-M	336(Z),sh,443,469	0.30
15	29.3	NI-M	336(Z),sh,441,468	0.35
16	29.7	*15-Z-Phytoene	285	
17	30.8	NI	sh,445,469	
18	31.7	*Phytofluene-1	331,346,364	
19	33.9	*β-cryptoxanthin	423,450,479	
20	34.2	Phytofluene-2 CC	332,348,364	
21	35.9	NI	327(Z), sh,445,472	
22	36.6	Z-ζ-carotene	295(Z),376,398,422	0.20
23	41.3	*ζ-carotene	378,399,424	
24	42.2	*β-carotene	426,451,473	
25	67.5	**All- E*-lycopene	445,472,502	

[a] *, identified using authentic standards; NI, not identified; M, only identified in mandarin samples; [b] Intensity of *Z* peak as ratio D_B/D_{II}.[32]

Table 2. Concentration of carotenoids (ng/g fresh weight) in pulp and freshly prepared juices and their corresponding bioaccessible fractions (ng/g fresh weight) from Washington Navel (N) and red-fleshed Cara Cara (CC) oranges.

	N (pulp)		N (juice)		CC (pulp)		CC (juice)	
Carotenoid	**Total**	**Bioaccessible**	**Total**	**Bioaccessible**	**Total**	**Bioaccessible**	**Total**	**Bioaccessible**
Phytoene	645±13a	56±14A	198±82b	45±1A	80664±3900c	3308±146B	13698±2030d	2675±251B
Phytofluene	86±34a	Tr.	23±9a	N.D.	11347±1347b	454±107A	577±42c	473±13A
Lycopene	N.D.	N.D.	N.D.	N.D.	9896±667b	74±5A	1908±161a	45±7B
β-carotene	N.D.	N.D.	N.D.	N.D.	171±12b	10±4A	86±4a	20±4B
β-Cryptoxanthin	565±15b	40±7A	382±69a	10±3B	207±3c	21±3C	219±2a	35±2A
Zeaxanthin	81±20a	Tr.	53±17ab	Tr.	Tr.	Tr.	34±1b	Tr.
Antheraxanthin	1109±63b	22±5A	301±108a	Tr.	681±176c	29±4A	317±61a	23±4A
All-*E*-Violaxanthin	764±69b	39±6A	281±63a	Tr.	263±49a	25±7A	286±19a	25±2A
9-*Z*-Violaxanthin	6514±185b	154±45A	3020±502ac	17±4B	3770±118c	136±5A	2130±203a	123±24A
Total carotenoids	**9764±347**c	**332±50**A	**4262±698**a	**77±5**B	**107125±6363**d	**4069±2**C	**19345±2591**b	**3430±277**D

Data are mean ± SD (n=4). Different lowercase letters (total content) and uppercase letters (bioaccessible fractions) on the same line represent significant differences ($p<0.05$) for a carotenoid among samples. Tr, Traces, values below 10 ng. N.D., not detected.

Table 3. Concentration of carotenoids (ng/g fresh weight) in pulp and freshly prepared juices and their corresponding bioaccessible fractions (ng/g fresh weight) from Clementine mandarins freshly harvested (M) and stored for 5 weeks at 12ºC (M12).

	M (pulp)		**M (juice)**		**M12 (pulp)**		**M12 (juice)**	
Carotenoid	**Total**	**Bioaccessible**	**Total**	**Bioaccessible**	**Total**	**Bioaccessible**	**Total**	**Bioaccessible**
Phytoene	1011±130a	205±25A	1280±37a	167±15A	1164±75a	344±93A	1278±100a	172±15A
Phytofluene	N.D.	N.D.	551±48	N.D.	N.D.	N.D.	N.D.	N.D.
β-Carotene	Tr.	Tr.	28±2a	Tr.	361±7b	109±3A	258±7c	46±1B
β-Cryptoxanthin	3782±76c	771±238A	3046±110a	200±13B	6518±63d	1872±69C	5396±471bd	1092±91A
Zeaxanthin	241±20c	34±6A	34±3a	Tr.	294±16d	80±6B	82±11b	54±7C
Antheraxanthin	1165±26c	137±29A	282±1a	28±5B	1432±34d	258±20C	400±28b	136±22A
All-*E*-Violaxanthin	580±20b	50±9A	99±24a	Tr.	815±7c	108±13B	120±26a	57±6A
9-*Z*-Violaxanthin	2627±86b	218±59A	535±10a	36±4B	3348±39c	417±12C	635±75a	199±14A
Total carotenoids	**9424±211**c	**1428±375**A	**5858±43**a	**453±22**B	**13503±548**d	**3190±212**C	**8169±400**b	**1847±129**A

Data are mean ± SD (n=4). Different lowercase letters (total content) and uppercase letters (bioaccessible fractions) on the same line represent significant differences ($p<0.05$) for a carotenoid among samples. Tr, Traces, values below 10 ng. N.D., not detected.

Electronic Supplementary Information

ESI Fig. 1S Distribution of individual carotenoids, as percentage of total carotenoid content, in pulp and freshly hand-squeezed juice of Washington Navel (N) and red-fleshed Cara Cara (CC).

ESI Fig. 2S Distribution of individual carotenoids, as percentage of total carotenoid content, in pulp and freshly hand-squeezed juice of Clementine mandarins (M) and Clementine mandarins stored for 5 weeks at 12 ºC (M12).

Fig. 1

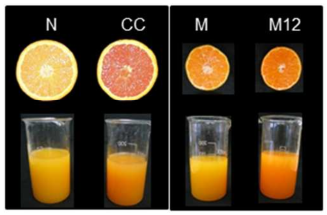

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Fig. 2

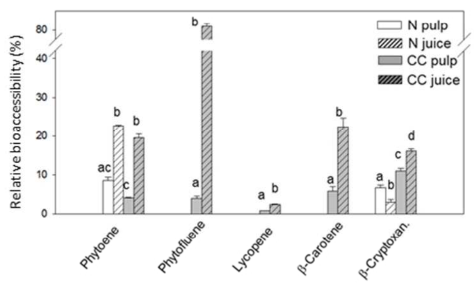

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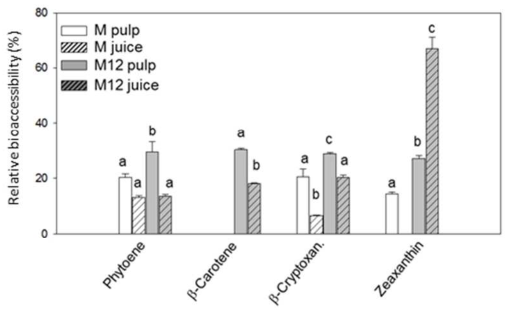
Fig. 3



254x190mm (96 x 96 DPI)