Impact of thermal treatments on the bioaccessibility of phytoene and phytofluene in relation to changes in the microstructure and size of orange juice particles

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HIGHLIGHTS

- Phytoene, phytofluene (PTF) and ζ-carotene behave similarly to thermal treatments.
- Phytoene and PTF have higher bioaccessibility than some carotenes and xanthophylls.
- TEM images show great disruptions of cell structures with the thermal treatments.
- Pasteurized juices have higher carotenoid bioaccessibility than frozen/thawed juices.

ABSTRACT

The interest in the colourless carotenoids phytoene and phytofluene is expanding. In this study their bioaccessibility from thermally treated Pinalate orange juices, which contains high concentrations of these carotenoids, was evaluated in relation to microstructural and particle size changes. Other carotenoids were also considered for comparison. Fresh, pasteurized, and ultrafrozen juices thawed at room temperature (UF-RT), in microwave oven (UF-MW), and in the fridge (UF-FG) were investigated. Colourless carotenoids suffered less and higher degradation as a result of ultrafreezing and pasteurization, respectively, than xanthophylls. In the fresh juice the carotenoid with the highest bioaccessibility was phytoene (10%), followed by zeaxanthin (9%) and phytofluene (8%). Total carotenoid bioaccessibility followed the order: Pasteurized > UF-MW > UF-RT > UF-FG > Fresh. Thermal treatments decreased the particle sizes and ruptured the cell structures and hence increased the bioaccessibility. The best source of bioaccessible colourless carotenoids was UF-MW (0.93 mg/250 mL).

KEYWORDS

In vitro digestion; particle size distribution; pasteurization; transmission electron microscopy; ζ-carotene.

ABBREVIATIONS

Conjugated double bonds (cdb)

Fresh juice (FRESH)

High performance liquid chromatography (HPLC)

Methanol (MeOH)

Methyl *tert*-butyl ether (MTBE)

One-way analysis of variance (ANOVA)

Particle size distribution (PSD)

Pasteurized juice (PAST)

Phytoene (PT)

Phytofluene (PTF)

Potassium hydroxide (KOH)

Strokes per minute (spm)

Transmission electron microscopy (TEM)

Ultrafrozen juice with thawing at room temperature (UF-RT)

Ultrafrozen juice with thawing in the microwave oven (UF-MW)

Ultrafrozen juice with thawing in the fridge (UF-FG)

1. Introduction

Carotenoids are a wide family of isoprenoid compounds with beneficial health properties (Kulczyński, Gramza-Michałowska, Kobus-Cisowska, & Kmiecik, 2017) and only a few of the more than 700 natural carotenoids have been studied in depth (Britton, Liaaen-Jensen, & Pfander, 2009). Phytoene (PT) and phytofluene (PTF) have been largely ignored for a long time as compared to others but are currently being a focus of major interest for the scientific community. In fact, some recent reviews have been published focusing in the importance of these two carotenoids in the diet as well as in their potential interest in the context of health promotion and cosmetics (Engelmann, Clinton, & Erdman, 2011; Meléndez-Martínez, Mapelli-Brahm, & Stinco, 2018; Meléndez-Martínez, Mapelli-Brahm, Benítez-González, & Stinco, 2015). PT and PTF are present in a wide variety of fruits and vegetables and their juices, and hence are among the predominant carotenoids in the diet (Biehler et al., 2012; Mapelli-Brahm, Corte-Real, Meléndez-Martínez, & Bohn, 2017). It is well known that tomato and tomato-based food products have a high concentration of both compounds (Khachik et

al., 2002). However, another source rich in these colourless carotenoids has recently been characterized, specifically the orange Pinalate (Lado et al., 2015; Rodrigo, Marcos, Alférez, Mallent, & Zacarías, 2003). This orange is a spontaneous mutant derived from the ordinary Navelate orange with a partial blockage at the ζ-carotene desaturation which seems to be the cause of the accumulation of these lineal carotenes. The flavedo of the Pinalate orange has one of the highest concentrations of PT in fruits described so far (Lado et al., 2015) and also contains very high concentrations of PTF and ζ-carotene (Rodrigo et al., 2003). However, it is important to consider that the beneficial actions of carotenoids and other species do not only depend on how much we ingest, but also on other factors, like for instance their bioavailability. Before their absorption, carotenoids have to be incorporated into mixed micelles to become absorbable for the intestinal enterocytes. It is widely accepted that the bioaccessibility of a given lipophilic compound is the amount of such compound that is released from the food matrix and incorporated into mixed micelles with respect to the initial amount present in the matrix. *In vitro* methods have been widely used to determine the bioaccessibility of carotenoids and to estimate their bioavailability (Estévez-Santiago, Olmedilla-Alonso, & Fernández-Jalao, 2016; Ornelas-Paz, Failla, Yahia, & Gardea-Bejar, 2008; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014). The bioaccessibility of PT and PTF has not been as widely studied as that of other dietary carotenoids but there are several articles that demonstrate their high bioaccessibility compared to that of other carotenes and even to some xanthophylls (Jeffery, Turner, & King, 2012; Mapelli-Brahm et al., 2017; Rodrigo, Cilla, Barberá, & Zacarías, 2015; Rodrigues, Chitchumroonchokchai, Mariutti, Mercadante, & Failla, 2017). Thermal treatments are widely used in the food industry for preservation (Ibarz, Pagán, & Garza, 1999) but these processes can affect the bioaccessibility of carotenoids and lead to the degradation

of some of them (Aschoff et al., 2015). Thus, thermal treatments can produce changes in food colour and be detrimental for the nutritional value (Palmero, Lemmens, Hendrickx, & Van Loey, 2014). Within this context, the main goals of this study were two. On the one hand, to understand how different thermal treatments of Pinalate orange juice affect the stability and the bioaccessibility of the colourless carotenoids phytoene and phytofluene and compare the results with those of other carotenoids present in the orange juice. On the other hand, as the thermal treatments can disrupt the food matrix and hence, enhance the release of the carotenoids and increase their bioaccessibility (Hof van het, West, Weststrate, & Hautvast, 2000), the effects of the changes in particle size distribution (PSD) and plastids integrity (assessed by transmission electron microscopy (TEM)) were also evaluated.

2. Materials and methods

2.1. Standards and reagents

HPLC solvents, i.e. methanol (MeOH) and methyl *tert*-butyl ether (MTBE), were of HPLC grade and were acquired from Panreac (Barcelona, Spain). The necessary reagents to obtain the images with the electronic transmission microscope were purchased from Ted Pella, Inc. (Redding, USA). Porcine bile extract, pepsin (porcine, 367 units/mg solid, measured as TCA- soluble products using hemoglobin as substrate) and pancreatin (porcine, 8x USP specifications of amylase, lipase and protease) were acquired from Sigma-Aldrich (Bornem, Belgium). All reagents were of analytical grade or higher.

2.2. Samples

The mutant Pinalate orange was chosen for this study due to its large accumulation in colourless carotenoids. It has been found that the flavedo and pulp of this orange are

rich in PT, PTF and ζ-carotene (Lado et al., 2015; Rodrigo et al., 2003). However, to the best of our knowledge, the juice has not been characterized. Mature Pinalate orange fruits (*Citrus sinensis* L. Osbeck), were harvested at full mature stage (11.9 ºBrix) in January 2016, from adult trees grown at The Citrus Germplasm Bank at the *Instituto Valenciano de Investigaciones Agrarias* (IVIA, Moncada, Valencia, Spain). All analyses were made with the same primary juice, which was obtained by manually squeezing representative orange replicates. Five aliquots (fresh samples) were stored in the fridge under nitrogen until analysis (one day). Pasteurization was carried out by immersion of 50 mL plastic tubes containing 20 mL of juice in a water bath at 120 ºC. After 30 seconds at 90 ºC, the tubes were immediately cooled in an ice bath until the samples reached 1.8 ºC. Pasteurized juices were stored under nitrogen atmosphere in the fridge until their analysis a few hours later. Ultrafreezing was carried out by direct immersion of 50 mL plastic tubes containing 20 mL of juice in liquid nitrogen for two minutes. The ultrafrozen samples were stored at - 80 ºC until their analysis. These were performed no more than three days later, after their thawing. The ultrafreezing and thawing treatments evaluated were the same considered in a previous study (Stinco, Fernández-Vázquez, Heredia, Meléndez-Martínez, & Vicario, 2013). On the day of the analysis, five replicates of the ultrafrozen juices were thawed at room temperature (avoiding light), another five were thawed in a fridge (4 ºC) and the last five were thawed in a microwave oven at 800 watts during 20 s, these latter conditions not leading to increases of temperature. Thus, five samples were analysed, fresh juice (FRESH), pasteurized juice (PAST) and ultrafrozen juices with thawing at room temperature (UF-RT), in the microwave oven (UF-MW) and in the fridge (UF-FG). FRESH was taken as reference sample in order to study the effect of the different thermal treatments on the

colour and particle size distribution of the juice and on the concentration and bioaccessibility of the carotenoids.

2.3. Transmission electron microscopy

Fresh juice, processed juices and pulp were analysed by TEM. Regarding the juices, 1.5 mL of juice was centrifuged at 18000 *g* for 5 min at 4 ºC in 2 mL vials (microfuge 22R, Beckman Coulter, Krefeld, Germany) and the aqueous phase was discarded. In the case of the pulp, few juice vesicles were manually cut by using a blade and were introduce in a 2 mL vial. Immediately, 1 mL of modified Karnovsky fixative (0.5% glutaraldehyde, 2.5% formaldehyde) was added to each vial. The cells were fixing during few hours in darkness and then centrifuged at 18000 *g* for 15 min at 4 ºC. The upper phase was discarded and the pellet was rinsed three times with 0.1 M sodium cacodylate buffer (pH 7.4). Then, the post-fixation was performed with 1% osmium tetroxide in the buffer for 1 h at 25 ºC. The sample was washed for 20 min at 4 ºC with distilled water and then stained with 2% aqueous solution of uranyl acetate for 2 h at 25 ºC. Dehydration was made in an acetone series (50, 70, 90, 100%). Subsequently, the sample was embedded in Spurr resin, following a gradual procedure with different ratios of acetone/spurr. Polymerization was carried out overnight at 70 °C. Ultrathin sections of 70-100 nm were obtained by cutting semithin sections with an ultramicrotom (Leica UC7, Wetzlar, Germany). These ultrathin sections were examined with a Zeiss Libra 120 transmission electron microscope (Oberkochen, Germany) equipped with a SSCCD digital camera.

2.4. Epifluorescence microscopy

The pulp and juice of the Pinalate orange were observed under a BX61 motorized epifluorescence microscope (Olympus, Tokio, Japan) in order to detect phytofluene. The pulp and juice of the parental Navelate were used as a reference. Fluorescence

microscopy allows for identifying specific fluorescent substances, named fluorochromes, by observing their characteristic emission properties when illuminated with radiation of appropriate wavelength. PTF, upon being excited with near-UV light (300 - 400 nm), emits light at approximately 510 nm and therefore can be detected by fluorescence microscopy (reviewed in Meléndez-Martínez et al., 2015). The arrangement of optical components of this microscope allows the illumination from above of the sample and therefore offers a high signal-to-noise ratio. The microscope was equipped with a mercury vapour lamp as light source (X-Cite 120PC) and with 10×/0.4 and 20×/0.75 UPLANAPO objective lenses. The images were acquired with an Olympus camera with a CellSens Dimension Software. An U-MNU2 filter (exciting filter BP 360 - 370 nm, emission filter BA420, and dichromatic mirror DM 400 nm) were applied. To obtain the images one drop of juice or one small piece of a cut vesicle juice was spotted on a microscope slide glass (26 × 76 mm) and a microscope cover glass (18 × 18 mm) was used to cover the sample. The use of a mounting medium was not necessary.

2.5. Particle Size Distribution

Particles scatter light in all directions with an intensity pattern, which is dependent on particle size. On the basis of this principle, the particle size distribution (PSD) of the juices was determined by laser diffraction using a Mastersizer 3000E equipped with a He-Ne laser (Malvern Instruments, Inc., Worcs, U.K.). The Mie model of light scattering recommended by ISO13320-1 November 1999 was used. This model assumes that particles are uniform spherical particles, which are illuminated by a plane wave of infinite extent with a known wavelength (633 nm). The optical properties of the sample and of the surrounding medium should be known in this model; the refractive index and absorption rate of the cloud particles for orange juice is 1.73 and 0.1,

respectively, while distilled water has a refractive index of 1.33 (Corredig, Kerr, & Wicker, 2001). To measure the PSD, an aliquot of 2.5 mL of juice was introduced into the sampling unit of the Mastersizer. The sample was pumped through the optical cell using a stirrer rotating at 2000 rpm and diluted with approximately 100 mL of distilled water to achieve an obscuration level of between 10-20%. The analysis report provided the frequency distribution graph, the surface area (A_S), the standard percentiles (D_v (10), D_v (50) and D_v (90)), and the volume- and area- based mean diameters ($D_{[4,3]}$ and $D_{[3,2],}$ respectively). D_v (10), D_v (50) and D_v (90) are the sizes in microns at which 90, 50 and 10% of the sample is smaller and 10, 50 and 90% is larger, respectively. $D_{[4,3]}$ and $D_{[3,2]}$ are defined by the following equation:

$$D_{[4,3]} = \sum_i n_i d_i^4 \Big/ \sum_i n_i d_i^3 \qquad \text{(Equation 1)}$$

$$D_{[3,2]} = \sum_i n_i d_i^3 \Big/ \sum_i n_i d_i^2 \qquad \text{(Equation 2)}$$

where n_i is the number of particles of diameter d_i.

2.6. Colour measurement

The reflectance spectra were measured using a CAS 140 B spectroradiometer (Instrument Systems, Instrument Systems, Munich, Germany) in the visible region (380–770 nm) in 2-nm steps. The D65 standard illuminant, corresponding to the natural daylight, and 10 ° Observer were assumed (CIE 1978). The spectroradiometer was equipped with a Top 100 telescope optical probe, a Tamron zoom mod. SP 23 A (Tamron USA, Inc., Commack, NY, USA), and as external light source a white light 150 W metal halide lamp Phillips MHN-TD Pro (12,900 lumen, 4200 K colour temperature). A 10 mm path length cuvette filled with distilled water against a white

background was used as a blank. The colour parameters of the uniform colour space CIELAB were obtained directly from the reflectance spectra from the apparatus. The CIELAB colour space is a coordinate cartometer system defined by three colorimetric coordinates, i.e. L^* (lightness), a^* (ranging from green to red) and b^* (ranging from blue to yellow). The hue angle (h_{ab}, the qualitative expression of colour) and the chroma (C^*_{ab}, the quantitative expression of colourfulness) are defined from these coordinates. The total colour differences (ΔE^*_{ab}) between the fresh juice sample (as a reference) and the rest of the samples were calculated using the following formula:

$$\Delta E^*_{ab} = ((L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2)^{1/2} \qquad \text{(Equation 3)}$$

In the preceding formula, subscript 1 refers to the fresh juice while the subscript 2 refers to the sample of interest in each case.

2.7. Total Soluble Solids (°Brix) and pH

The maturity stage of the samples was measured as ºBrix with an ABBE refractometer (WYA-S, Biotech) at 20 ºC. The pH was measured with a pH-meter (GLP 21 pH-meter, Crison, Barcelona, Spain).

2.8. In vitro digestion

The *in vitro* gastro-intestinal digestion model was based on the protocol described by Stinco et al. (2012) with some modifications. Three grams of juice and 5.4 mL of aqueous saline solution (140 mM NaCl/5 mM KCl) were mixed in a 50-mL plastic tube. The pH was adjusted to 2 by adding 0.1 M HCl. All samples were complemented with distilled water up to a final volume of 12.4 mL. Then, 0.6 mL of pepsin solution (40 mg/mL in 0.1 M HCl, prepared the day of usage) was incorporated into the mixture. To simulate the gastric digestion, the samples were incubated for 1 h at 37 ºC in a Max

Q5000 shaker (Labware, Madrid, Spain) with reciprocating motion at 100 strokes per minute (spm). Afterward, samples were maintained in ice few minutes to inactivate the enzymes. The pH was then increased to 6.9 by adding 1 M NaOH and the solution was made up to a final volume of 15 mL with distilled water. Subsequently, 0.75 mL of a bile extract and pancreatin solution (2 mg/mL pancreatin and 12 mg/mL bile in 0.1 M $NaHCO_3$, prepared the day of usage) was added. Samples were incubated for 2 h at 37 ºC in the shaker at 100 spm to mimicking the intestinal digestion. Once completed the digestion, samples were centrifuged at 5000 *g* for 20 min at 4 °C. The supernatant was filtered through a 0.22 µm nylon membrane (Millipore Iberica S.A., Madrid, Spain) into a new 50 mL plastic tube. The mixed micelle fractions obtained, i.e. the bioaccessible fraction, were flushed with nitrogen and stored at -80 ºC until carotenoids extraction (one day).

2.9. Carotenoids analysis

Six hundred microliters of diethyl ether were added to 0.5 g of juice in a 2-mL plastic tube. The mixture was vortexed and ultrasonicated (Ultrasons, JP Selecta, Barcelona, Spain) for 2 min. To promote phase separation, the sample was centrifuged (Microfuge 22R, Beckman Coulter, Madrid, Spain) at 18000 *g* for 5 minutes at 4 °C. Then, the upper phase containing the carotenoids was transferred to another 2 mL plastic tube. The matrix was re-extracted twice with 600 µL of diethyl ether and the organic phases were combined together. The pooled ether phase was concentrated to dryness in a rotary evaporator at 30 ºC (Eppendorf Concentrator Plus, Hamburg, Germany). The extract was saponified by adding 600 µL dichloromethane and 600 µL methanolic potassium hydroxide (KOH) (30%, w/v) and the mixture was maintained 30 min with mechanical shake (Gyromini Nutating 3-D Mixer, Labnet, Madrid, Spain) in the dark under nitrogen. The saponification time and KOH concentration were chosen

after carrying out some preliminary tests. Saponification tests included KOH at 30 or 40% in MeOH and saponification times from 30 min to overnight. After saponification, the organic phase was washed with distilled water until neutral pH of the waste water. Finally, the organic phase was concentrated to dryness by rotary evaporation at 30 ºC. The extracts were kept at -80 ºC under a nitrogen atmosphere until the HPLC analysis.

The extraction of carotenoids from digesta was carried out similarly with the following minor changes. Ten millilitres of diethyl ether and ten millilitres of NaCl were added to the entire mixed micelle fraction. The mixture was vortexed for 1 min and centrifuged for 5 min at 3280 *g*. The upper layer was transferred to a 15 mL plastic tube. The extraction was repeated twice more by adding 5 mL of ethyl ether at each step. Saponification of the dry extract was carried out in the same manner but with 2 mL of KOH (30% MeOH) and 2 mL of dichloromethane.

2.10. HPLC-DAD analysis

The extracts were analysed by reverse-phase HPLC (Agilent 1260 system, Waldbronn, Germany) with UV/VIS diode array detector. A C_{30} YMC column (3 μm, 150 cm × 4.6 mm) and a C_{30} YMC pre-column (2.7 μm, 50 mm × 4.6 mm) (Wilmington, NC, USA) were used. The chromatographic method was similar to that published by Stinco et al. (2012) with minor modifications. Thus, the mobile phase consisting of MeOH (A), MTBE (B) and Milli-Q quality water (C). The linear gradient elution was: 0 min, 90% A + 5% B + 5% C; 5 min, 95% A + 5% B; 10 min, 89% A + 11% B; 16 min, 75% A + 25% B; 20 min, 40% A + 60% B; 22.5 min, 15% A + 85% B; 25 min, 90% A + 5% B + 5% C. The run time was 27 min during which the flow rate was 1 mL/min and the column was kept at 30 °C. The detector was set at 286 nm for the detection of phytoene, at 350 nm for phytofluene, at 410 nm for ζ-carotene, and at 450 nm for the rest of the carotenoids. Prior to the injection the samples were dissolved in

ethyl acetate. Juice extracts were dissolved in 100 µL and 7 µL were injected while the digesta extracts were dissolved in 30 µL and 20 µL were injected. The identification of the carotenoids was made by comparison of their spectroscopic and chromatographic features with those of the collection of standards of the Food Colour and Quality Lab.. External calibration was used for quantification.

2.11. Calculations and statistical analysis

All analyses were carried out in triplicate except the *in vitro* digestions and the colour analyses that were performed in quintuplicate. The results shown in the text and the tables were expressed as mean values ± standard deviations. In the graphics, the error bars represent ± standard deviation. Data processing was performed using the IBM SPSS Statistics 20® software (SPSS Inc., 2012). Shapiro-Wilk and Levene tests ($P \leq 0.05$) were used to verify the normality and homoscedasticity, respectively. One-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test was performed in order to detect significant differences among means. When the results did not allow carrying out parametric studies, the comparisons were carried out following the *post hoc* non-parametric T2-Tamhane test. Differences were considered statistically significant at P value ≤ 0.05 (2-sided).

Relative bioaccessibility was calculated as the percentage of carotenoid content that remained in the micellar aqueous fraction after centrifugation and filtration in relation to the respective initial content in the original non-digested matrix. To estimate the bioaccessible content the relative bioaccessibility and the initial concentration in the matrix were taken into account. In this sense, it was regarded as the amount of carotenoid that can be potentially absorbed per ration of food.

3. Results and discussion

3.1. Transmission electron microscopy (TEM)

TEM images of the pulp showed that mature Pinalate orange did not contain chloroplasts and was rich in chromoplasts containing round vesicles, achlorophyllous membranes, plastoglobuli, and some starch grains. Thus, in Figure 2-A two intact chromoplasts can be easily seen. Most of the chromoplasts were rich in round vesicles which were larger than the plastoglobuli, in agreement with the TEM results of Lado et al. (2015). These vesicles seem to contribute to the large accumulation of linear carotenes in this mutant (Lado et al., 2015). In the fresh juice, vesicles, plastoglobuli and starch grains fairly intact were observed, although in some cases outside the chromoplasts which were broken during juicing. In general, it can be observed a gradual degradation of the cell and plastid structures in the following order: PULP, FRESH, UF-FG, UF-RT, UF-MW and PAST. Thus, while fresh juice still contained a large number of intact plastid internal structures, in the PAST the great disruption of cell material was quite evident and were almost devoid of intact suborganellar structures. In agreement with our results, Gupta et al. (2011) found intact cellular components in a freshly extracted tomato juice while cellular components were indistinguishable in a pasteurized juice (100 ºC, 10 min, 0.1 MPa). In the ultrafrozen juices, the ice crystals could be the cause of the rupture of the cell structures (Leong & Oey, 2012).

3.2. Epifluorescence microscopy

The pulp and juice were observed under an epifluorescence microscope in order to evaluate the distribution of PTF in these matrices. Both, pulp and juice of the Pinalate orange showed fluorescence, being more easily detected and concentrated in the pulp (Figures 1-A and 1-B). In the juice the fluorescence was more scattered, indicating a greater dispersion of phytofluene, which could have an impact in its release during digestion. These images were compared to those of the parental Navelate orange which

only contain a small amount of PTF (Stinco, Escudero-Gilete, Heredia, Vicario, & Meléndez-Martínez, 2016) in comparison with that of Pinalate (Table 1). Fluorescence was hardly observed in the pulp of Navelate orange (Figures 1-C and 1-D). From these results, it can be inferred that epifluorescence microscopy could be an efficient, rapid and reliable technique to detect PTF in orange juices.

3.3. Particle Size Distribution (PSD)

Regarding the PSD, all parameters analysed followed the decreasing order: FRESH > UF-FG > UF-RT > UF-MW > PAST, being the differences between FRESH and PAST in all cases statistically significant (Table 2). The volume- and area- based mean diameters ($D_{[4,3]}$ and $D_{[3,2]}$, respectively) were 14.4% and 12.5% respectively lower in PAST than in FRESH (Table 2). The cloud size distributions were bimodal in all juices and, as can also be seen in Figure 3, FRESH was the juice with the highest percentage of particles with a bigger size and with the lowest percentage of particles with a lower size followed by UF-FG, UF-RT, UF-MW and PAST. That is, PAST was the juice with the lowest particle size. PSD results of FRESH were similar to those found by Stinco et al. (2012). Other researches have shown conflicting results on the change in the particle size due to the pasteurization of fresh orange juices (Leizerson & Shimoni, 2005; Stinco et al., 2012). To the best of our knowledge, the effect of freezing and thawing on the particle size of orange juices is not well described in the literature. However, it should be pointed out that, in many articles, juices which are regarded as fresh juices were in fact frozen at some point. This is an important aspect to take into account, as it has been proven that this process can have important effects in the PSD (Table 2) and therefore in the colour and bioaccessibility of the carotenoids (sections 3.4 and 3.7).

3.4. Colour measurement

Colour is an attribute of paramount importance for the consumers and in Pinalate juice it could be strongly influenced by its peculiar profile of carotenoids. The main coloured carotenoid in Pinalate juice, i.e. ζ-carotene (Table 3), has a b^* and C^*_{ab} values much lower than those of the main carotenoids in common orange juices, i.e. violaxanthin and β-cryptoxanthin (Meléndez-Martínez, Britton, Vicario, & Heredia, 2007; Stinco et al., 2016). This may be one of the main reasons why fresh Pinalate juice has a significantly lower b^* and C^*_{ab} values, i.e. 30 and 32 respectively, compared to those average values found in common orange juices, i.e. approximately between 59 and 62 and between 50 and 61, respectively (Fernández-Vázquez, Stinco, Meléndez-Martínez, Heredia, & Vicario, 2011; Stinco et al., 2016). As a result, Pinalate juice has a light-yellowish colour, which correlates well with that of ζ-carotene (Meléndez-Martínez et al., 2007), while common orange juices are more orange. On the other hand, the high content of colourless carotenoids PT and PTF and the low content of xanthophylls could contribute to the pale colour of the Pinalate juice. The colour attributes a^* and b^* were positive in all samples.

The colour of FRESH was affected by the thermal treatments. Considering the significant decrease in the lightness value of PAST in comparison to that of FRESH (Table 3) it can be concluded that pasteurization caused a darkening of the juice. Regarding C^*_{ab} and h_{ab} parameters, no statistically significant differences were found with any of the treatments. These results are in agreement with those of a study with ultrafrozen juice of Valencia late oranges thawed under conditions analogous to those of this study (Stinco, Fernández-Vázquez, Heredia, Meléndez-Martínez, & Vicario, 2013). However, contradictory results have been found in the literature regarding the changes in the colour parameters of thermally-treated orange juices (Ortiz et al., 2017; Stinco et al., 2016; Stinco et al., 2013; Wibowo et al., 2015). These differences may be due to

some extent to differences in the carotenoid levels of the oranges (Wibowo et al., 2015). However, it should be noted that changes in the concentration of ascorbic acid can also influence the colour of orange juices (Meléndez-Martínez, Vicario, & Heredia, 2009; Roig, Bello, Rivera, & Kennedy, 1999) and that orange juices browning could be due to the oxidation of phenols to quinones (Eissa, Fadelb, Ibrahim, Hassan, & Elrashid, 2006). ΔE* is a good parameter for understanding how observers perceive colour differences and it is considered that, ΔE* over 2.8 CIELAB units can be perceived by even inexperienced observers (Melgosa, Pérez, Yebra, Huertas, & Hita, 2001). Differences above this threshold were found in UF-FG, UF-MW, and PAST (Table 3). The differences found in the ultrafrozen juices are very similar to those found in other studies (Cinquanta, Albanese, Cuccurullo, & Di Matteo, 2010; Stinco et al., 2013). Among the thermal treatments, pasteurization generated the greatest colour difference (ΔE^*) with respect to FRESH while the smallest difference occurred in the UF-RT (Table 3). This could be related to the fact that UF-RT was the juice with the lowest degradation of carotenoids and PAST the one that suffered the greatest degradation (Supplementary Table 1).

3.5. Carotenoid profile in the fresh orange juice

Violaxanthin, antheraxanthin (cyclic epoxycarotenoids), zeaxanthin (cyclic dihydroxycarotenoid), β-carotene (cyclic carotene) and three linear carotenes, i.e. ζ-carotene, PT and PTF, were detected. That is, three xanthophylls and four carotenes. β-Carotene is one of most studied carotenoids due to its high provitamin A activity and zeaxanthin has also been extensively studied for its role in the human macula (Johnson et al., 2000). On the other hand, although PT, PTF and ζ-carotene are found in human serum they are very poorly studied carotenoids. The most abundant carotenoid was ζ-carotene followed by PTF and PT (Table 1). Other researchers have found a higher

concentration of PT with respect to that of ζ-carotene in the pulp of Pinalate (Lado et al., 2015). This difference could be due to the possible difference in the degree of release between the carotenoids when juicing, and this could, in turn, be due to the difference in the subcellular structures where each carotenoid accumulates (Rodrigo, Cilla, Reyes, & Zacarías, 2015). Regarding the isomers, 4 of ζ-carotene and PTF, 2 of PT, and only the 9-*cis* isomer of violaxanthin and antheraxanthin were detected. In addition, four compounds were detected but could not been identified. As their retention times were similar to those of the common carotenoid esters, different saponification conditions were tested in order to be certain whether they were free carotenoids or not. The areas of their respective peaks were not altered with the different saponification times and KOH concentrations so it was concluded that they were free carotenoids. Taking into account that they could not be identified and that they were not one of the six carotenoids that represent more than 95% of the total carotenoids in human blood (Maiani et al., 2009), they were not included in the present study. However, their chromatographic and spectroscopic characteristics are summarized in Supplementary Table 2. The carotenoid profile found in the Pinalate juice is very different from that of other coloured common citrus fruits. In the latter, xanthophylls represent up to 80% of the total carotenoid content and linear carotenes account for no more than 20% (Rodrigo et al., 2003) while, in the Pinalate juice, ζ-carotene, PT and PTF together represented nearly 98% of the total carotenoids and only 1.2% of the carotenoids were xanthophylls (Table 1). Thus, while in juices of common varieties of orange the concentration of PT and PTF is approximately 1 and 0.35 mg/L respectively (Stinco et al., 2016), in Pinalate juice the concentration was 14 and 19 mg/L, respectively. Although Cara Cara is also considered an orange rich in these carotenoids, with reported values of 12 and 3 mg/L of

PT and PTF respectively (Stinco et al., 2016), the concentration is lower than that of Pinalate.

3.6. Carotenoid degradation during thermal treatments

The degradation of carotenoids is an important aspect for the food industry since it can affect the colour and the nutritional value of foods. Important percentages of degradation of carotenoids were observed across the different treatments (Supplementary Table 1). PT and PTF were the carotenoids which suffered the highest degradation during pasteurization, with approximately 67% of degradation. On the other hand, in all ultrafrozen juices, PT and PTF were more stable than xanthophylls (Supplementary Table 1). These results seem to indicate that the colourless carotenoids are less prone to degradation due to the combination of oxygen, enzymes and acid, but less heat stable than the xanthophylls. This difference in the behaviour of PT and PTF compared to the xanthophylls may be due to some extent to structural and physico-chemical differences, for instance their absence of terminal rings, differences in polarity, or the smaller number of conjugated double bonds, which is known to have a remarkable impact in the electron affinity and ionization energy of carotenoids (Martínez, Stinco, & Meléndez-Martínez, 2014). Likewise, they could be due, at least in part, to differences in their accumulation within the cell, like for instance the substructures they may deposit or in the aggregates that might be formed. However, it must be taken into account that orange juice is a complex matrix and that, to be able to obtain more specific conclusions about the degradation of PT and PTF as compared to other orange juice carotenoids, it seems more reasonable to carry out oxidation studies with carotenoid standards under identical conditions. Violaxanthin was the carotenoid which suffered the highest losses with the three types of ultrafreezing and thawing, with an average degradation of 76.5% among the different thawing conditions. This is

consistent with the fact that 5,6-epoxycarotenoids suffer a re-arrangement to their respective 5,8-furanoxide in acidic media (Meléndez-Martínez, Vicario, & Heredia, 2007).

Regarding total carotenoid concentration, FRESH was the juice with the highest content, i.e. 7.7 mg/100 g, while PAST was the juice with the lowest concentration, i.e. 2.6 mg/100g (Table 1), ca. 3-fold lower compared to FRESH. Significant differences were observed in total carotenoid content between fresh and ultrafrozen juices, although previous work did not find differences (Stinco et al., 2013). This differential behaviour may be related to the differences in carotenoid composition between standard sweet orange juices and Pinalate juice. The percentages of carotenoid degradation in the juices followed the order: UF-RT (32%), UF-FG (41%), UF-MW (42%) and PAST (67%) (Supplementary Table 1). Interesting, this increasing order agreed well with that of the degree of microstructural changes (section 3.2). In this sense, it seems reasonable to hypothesize that the greater the degradation of the cell structures, the more exposed the carotenoids are to the acid environment, oxygen, and enzymes, and so, the greater degradation they suffer. The greater degradation of carotenoids and plastids found in PAST could be due to the combination of the aforementioned factors and the heat, as it is well known that the oxidative degradation of carotenoids is stimulated by heat (reviewed in Rodríguez-Amaya & Kimura, 2004; Rodríguez-Amaya, 1999). Among the ultrafrozen juices, UF-FG suffered the highest loss of carotenoids, a result that is in good agreement with the fact that a slow defrosting causes greater carotenoid losses than a rapid thawing (reviewed in Rodríguez-Amaya & Kimura, 2004).

3.7. Carotenoid bioaccessibility

In all the juices, PT had a bioaccessibility higher than that of PTF and the bioaccessibility of both was higher than that of ζ-carotene. This order among the linear

carotenes might be due to the difference in the number of conjugated double bonds (cdb). In this regard, it could be argued that the higher number of cdb of ζ-carotene (7 cdb) in comparison with those of PTF (5 cdb) and PT (3 cdb) makes this carotene more rigid and prone to aggregation of their molecules, which could lead to a decrease in the bioaccessibility, similarly to what were found when comparing PT, PTF and lycopene (Mapelli-Brahm et al., 2017; Meléndez-Martínez, Paulino, Stinco, Mapelli-Brahm, & Wang, 2014; Rodrigo, Cilla, Barberá, et al., 2015). In all the samples, the bioaccessibility of the colourless carotenoids was even higher than that of the xanthophylls antheraxanthin and violaxanthin. These epoxycarotenoids are not found at detectable levels in human fluids or tissues (Khachik, 2006), while PT and PTF are common circulating carotenoids and have been found in several organs (reviewed in Meléndez-Martínez et al., 2015). The only carotenoid that showed greater bioaccessibility than the colourless carotenoids was zeaxanthin, although in all cases the difference with PT was not statistically significant. The high relative bioaccessibility of PT and PTF compared to other carotenoids is consistent with previous investigations. The colourless carotenoids showed bioaccessibilities higher than those of β-carotene, α-carotene and/or lycopene in carrot, papaya, different types of salads, apricot juice, grapefruit, melon, watermelon, tomato, and tomato juice (Jeffery et al., 2012; Mapelli-Brahm et al., 2017; Rodrigues et al., 2017), and it was found to be even higher than that of other xanthophylls such as β-cryptoxanthin, lutein and violaxanthin in certain matrices (Jeffery et al., 2012; Mapelli-Brahm et al., 2017).

Total carotenoid bioaccessibility followed the order: PAST (26%) > UF-MW (18%) > UF-RT (14%) > UF-FG (12%) > FRESH (8%). Thus, the pasteurization increased such parameter over 3-fold and the freezing followed by thawing with microwave over 2-fold. The same order was followed by all the carotenoids with the

exception of violaxanthin and antheraxanthin (Table 4). Thus, PAST was the juice with the highest bioaccessibility of PT and PTF, i.e. 32 and 27%, respectively, while the lowest was FRESH with 10 and 8% (Table 4), that is over 3-fold less for both carotenes. The differences in the bioaccessibility of each carotenoid and the total bioaccessibility between PAST and FRESH were statistically significant ($P < 0.05$). These results highlight the importance of the food matrix effect on the bioaccessibility. Since carotenoids must release from the food matrix in order to be incorporated into the mixed micelles, it seems reasonable to expect that the more the matrix is disrupted, the higher the bioaccessibility will be. In order to test this hypothesis, the particle size and the cell structures degradation of the samples were studied, and the results of both analyses confirmed this assumption. Thus, TEM images (Figure 2) showed the same increasing order in the degradation of the plastids among the samples (section 3.2) than the order found in the bioaccessibility. PSD results also agree with those of TEM and bioaccessibility, indicating that FRESH was the juice with the highest percentage of particles with a big size and the lowest percentage of particles with a low size, followed by UF-FG, UF-RT, UF-MW and PAST (section 3.3). Taken together, it could be concluded that the higher bioaccessibility found in PAST is due to the fact that the effect of heat during pasteurization causes greater matrix degradation than the ultrafreezing followed by thawing. On the other hand, it is considered that the higher the concentration of a given carotenoid in a matrix, the lower bioaccessibility, among other reasons because high concentrations could increase the possibility of molecular aggregation (reviewed in Borel, 2003; Mapelli-Brahm et al., 2017). Consistent with the above, it was found that, probably due in part to the degradation, the carotenoid concentration in PAST was significantly lower than that of FRESH (Table 1). Other authors have found increases in carotenoids bioaccessibility in orange juices with the

pasteurization (Aschoff et al., 2015). On the other hand, Stinco et al. (2013), also found higher carotenoid bioaccessibility when orange juices were subjected to ultrafreezing following to thawing in the microwave oven but this increase was not found when the juices were defrosting at room temperature or in the fridge.

In order to study whether the thermal treatments affect to the same extent the bioaccessibility of the different carotenoids, the increase in the bioaccessibility for each carotenoid with the thermal treatments, taking as the reference the respective bioaccessibility in the FRESH, was calculated (Supplementary Table 3). The percentages of increase in the bioaccessibility among the carotenes were very similar while in the xanthophylls these values were less homogeneous. This may be due in part to the greater difference with respect to the number and type of functional groups, i.e. epoxides and hydroxyls, and with this the greater difference in the polarity, found among the xanthophylls as compared to carotenes. Also the variety in the degree of esterification and in the fatty acids that may be involved in each ester could be one of the causes of this difference (Borel, 2003). The average increase in the bioaccessibility of the carotenes with the pasteurization was 238% while in the UF-FG the bioaccessibility was increased by only 49%. Interestingly, for all treatments, the bioaccessibility increase was higher in PTF than in PT.

The pH of the samples was measured as it is known that the pH can affect the transfer of carotenoids to mixed micelles during the digestion (reviewed in Reboul & Borel, 2011). However, no significant changes in the pH were found with the thermal treatments, being 3.8 the pH average (data not shown). In any case, the effect of possible differences in the acidity of the juices was expected to be counteracted by the pH reached during the gastric and intestinal digestion phases, which was homogeneous across samples.

3.8. Bioaccessible carotenoid content

Information on the bioaccessible carotenoid contents can be more meaningful as they represent the potentially absorbable amount of carotenoids. These data are summarized in Table 5. In all samples, ζ-carotene was the carotenoid with the highest bioaccessible content, followed by PT and PTF which showed virtually the same bioaccessible content. The intake of approximately one glass of FRESH, i.e. 250 mL, provided 0.7 mg of ζ-carotene and 0.4 mg of PT and PTF. With the exception of ζ-carotene, the amount of potentially absorbable PT and PTF that is provided with the intake of any of the juices is much higher than that of the rest of carotenoids. Thus, for example, the bioaccessible amount of PT or PTF is more than 100 times greater than that of violaxanthin.

Noteworthy is that neither the PAST, which presented the highest total carotenoid bioaccessibility, nor the FRESH, which exhibited the highest initial carotenoid concentration were the juices with highest content of bioaccessible carotenoids. Thus, the source that provided the highest quantities of bioaccessible PT, PTF, and ζ-carotene and the highest total carotenoid bioaccessible content was UF-MW, while the lowest was UF-FG. This clearly demonstrates the importance of using the bioaccessible content rather than the percentage of bioaccessibility and the concentration in the matrix, when it comes to evaluate the goodness of a food to raise the carotenoid status in humans. UF-FG was the only juice with an bioaccessible content of PT, PTF and total carotenoid lower than that of FRESH. In all the cases, the increase in the bioaccessible content with the thermal treatments was higher for PTF compared to PT, and this could mainly due to the higher increase in the bioaccessibility of PTF with the different thermal treatments.

4. Conclusions

This study provides information on the bioaccessibility and stability of three carotenoids, i.e. PT, PTF and ζ-carotene that, despite having been found in human plasma, have been very little studied. These carotenes are particularly abundant in the sweet orange cultivar Pinalate, being then an excellent system to analyse their bioaccessibility in orange juices, a common carotenoid source in the diet, and also to investigate the effects of the preserving juice treatments on these carotenes. These three compounds have a similar behaviour upon thermal treatments, that is, they suffered a similar degradation and presented similar bioaccessibility in the samples. However, the highest bioaccessibility in all the samples was that of the PT and the lowest was that of the ζ-carotene, and this could be mainly due to the differences in the number of conjugated double bonds. On the other hand, considering just the treatments and not the digestion, it seems that PT and PTF compared to xanthophylls are less prone to degradation due to the combination of oxygen, enzymes and acid, but less heat stable. Possible explanations for this are the lack of terminal ring, the lower number of conjugated double bonds, the lower polarity, and the sites and patterns of accumulation in the cells of the colourless carotenoids.

In summary, this study shows how pasteurization or ultrafreezing followed by thawing have a great impact on the colour of the orange juice and on the concentration and bioaccessibility of the carotenoids. Taking into account the particle size distribution and the cell structures degradation, it can be concluded that the increase in the bioaccessibility is mainly due to the degree of disruption that the matrix suffered with the thermal treatments. Although the thermal treatments tested generated carotenoids losses, the ultrafrozen juices which were thawed at room temperature or in the

microwave oven were better sources of bioaccessible phytoene, phytofluene, and total carotenoids than the fresh juice.

Thus, this study can be of interest to the functional food and nutricosmetics industries as phytoene and phytofluene are raising increasing awareness as evidence is accumulating that they may provide diverse health and cosmetic benefits.

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TABLES

Table 1. Carotenoid concentration (mg/100 g juice) of Pinalate fresh orange juice and Pinalate orange juices subjected to different thermal treatments

	Juice samples				
	FRESH	UF-RT	UF-FG	UF-MW	PAST
Phytoene	1.39 ± 0.14^{Ac}	1.00 ± 0.05^{Bb}	0.83 ± 0.06^{Bc}	0.79 ± 0.08^{BCb}	0.45 ± 0.10^{Cbc}
Phytofluene	1.81 ± 0.14^{Ab}	1.24 ± 0.07^{Bb}	1.08 ± 0.08^{Bb}	1.04 ± 0.10^{Bb}	0.59 ± 0.11^{Cb}
ζ-Carotene	4.35 ± 0.26^{Aa}	2.90 ± 0.19^{Ba}	2.59 ± 0.19^{Ba}	2.55 ± 0.25^{Ba}	1.47 ± 0.25^{Ca}

9-*cis*-Violaxanthin	0.05 ± 0.01^{Ad}	0.01 ± 0.00^{Bc}	0.01 ± 0.00^{Bd}	0.01 ± 0.00^{Bc}	0.02 ± 0.00^{Bc}
Zeaxanthin	0.02 ± 0.00^{Ad}	0.01 ± 0.00^{Bc}	0.01 ± 0.00^{Bd}	0.01 ± 0.00^{Bc}	0.01 ± 0.00^{Bc}
9-*cis*-Antheraxanthin	0.02 ± 0.00^{Ad}	0.01 ± 0.00^{Cc}	0.01 ± 0.00^{Cd}	0.01 ± 0.00^{Cc}	0.01 ± 0.00^{Cc}
β-Carotene	0.09 ± 0.00^{Ad}	0.07 ± 0.00^{Bc}	0.06 ± 0.00^{BCDd}	0.06 ± 0.01^{BCDc}	0.03 ± 0.00^{Ec}
Total	7.73 ± 0.55^{A}	5.24 ± 0.32^{B}	4.59 ± 0.33^{B}	4.47 ± 0.44^{B}	2.57 ± 0.47^{C}

FRESH, Fresh orange juice; UF-RT, ultrafrozen juice with thawing at room temperature; UF-FG, ultrafrozen juice with thawing in the fridge; UF-MW, ultrafrozen juice with thawing in the microwave oven; PAST, pasteurized juice. Values are the mean ± SD of 3 independent measures. Values within a column and within a row with different lowercase and uppercase letters respectively indicate statistically significant differences ($P < 0.05$).

Table 2. Particle size characteristics in Pinalate fresh orange juice and Pinalate orange juices subjected to different thermal treatments.

	Juice samples				
	FRESH	UF-RT	UF-FG	UF-MW	PAST
A_s	91.7 ± 2.5^{c}	105.1 ± 1.6^{b}	93.2 ± 3.1^{c}	110.0 ± 4.4^{ab}	115.4 ± 1.8^{a}
$D_{[3,2]}$	62.3 ± 1.7^{a}	54.4 ± 0.8^{b}	61.3 ± 2.0^{a}	52.0 ± 2.0^{bc}	49.5 ± 0.7^{c}
$D_{[4,3]}$	446.3 ± 8.3^{a}	382.0 ± 6.6^{c}	413.0 ± 4.6^{b}	351.7 ± 8.5^{d}	325.3 ± 5.5^{e}
D_v (10)	27.7 ± 1.4^{a}	22.6 ± 0.5^{b}	25.5 ± 0.9^{a}	22.1 ± 1.0^{b}	21.6 ± 0.3^{b}

D_v (50)	467.7 ± 10.2[a]	365.3 ± 14.0[c]	421.0 ± 6.6[b]	296.3 ± 18.9[d]	224.7 ± 11.7[e]
D_v (90)	844.3 ± 5.5[a]	812.0 ± 5.0[c]	829.3 ± 2.3[b]	780.3 ± 7.4[d]	764.0 ± 6.6[e]

As, specific surface area (m^2/kg); $D_{[3,2]}$, surface area-based mean diameter (µm); $D_{[4,3]}$, volume-based mean diameter (µm); D_v (10), D_v (50) and D_v (90), values of particle size below which there is 10%, 50% and 90% of sample volume, respectively (µm). FRESH, Fresh orange juice; UF-RT, ultrafrozen juice with thawing at room temperature; UF-FG, ultrafrozen juice with thawing in the fridge; UF-MW, ultrafrozen juice with thawing in the microwave oven; PAST, pasteurized juice. Values are the mean ± SD of 3 independent measures. Values within a raw with different lowercase letters indicate statistically significant differences ($P < 0.05$).

Table 3

CIELAB colour parameters of Pinalate fresh orange juice and Pinalate orange juices that were subjected to different thermal treatments.

	Colour parameters			
	L^*	C^*_{ab}	h_{ab}	ΔE^*
FRESH	86.6 ± 1.8[a]	31.83 ± 1.90[a]	88.78 ± 1.69[a]	-
UF-RT	85.1 ± 0.6[ab]	32.36 ± 1.42[a]	87.60 ± 1.90[a]	1.17 ± 0.15[c]
UF-FG	84.1 ± 0.6[ab]	34. 47 ± 1.43[a]	87.26 ± 1.75[a]	2.93 ± 0.51[b]

UF-MW	84.6 ± 0.4^{ab}	33.87 ± 0.82^{a}	87.75 ± 1.57^{a}	3.55 ± 0.60^{ab}
PAST	82.4 ± 0.5^{b}	31.24 ± 1.34^{a}	86.47 ± 1.84^{a}	4.15 ± 0.12^{a}

FRESH, Fresh orange juice; UF-RT, ultrafrozen juice with thawing at room temperature; UF-FG, ultrafrozen juice with thawing in the fridge; UF-MW, ultrafrozen juice with thawing in the microwave oven; PAST, pasteurized juice. Values are the mean ± SD of 5 independent measures. L^*, lightness; C^*_{ab}, chroma; h_{ab}, hue angle; ΔE^*, colour difference. ΔE^* was calculated taken the fresh juices as reference sample. Values within a column with different lowercase letters indicate statistically significant differences ($P < 0.05$).

Table 4. Carotenoid bioaccessibility (in percentage) of Pinalate fresh orange juice and Pinalate orange juices subjected to different thermal treatments.

	Juice samples				
	FRESH	UF-RT	UF-FG	UF-MW	PAST
Phytoene	10.26 ± 0.27^{Ca}	17.06 ± 1.54^{BCa}	14.18 ± 0.75^{BCab}	21.51 ± 2.42^{Bab}	31.97 ± 4.82^{Aab}
Phytofluene	7.76 ± 0.24^{Cbc}	14.74 ± 1.00^{Bab}	11.89 ± 0.74^{BCbc}	18.55 ± 1.96^{Bbc}	26.70 ± 4.51^{Ab}
ζ-Carotene	6.72 ± 0.31^{Dc}	13.41 ± 0.67^{BCab}	10.65 ± 0.74^{CDcd}	17.19 ± 1.71^{Bbc}	24.16 ± 4.26^{Ab}
9-*cis*-Violaxanthin	2.72 ± 0.39^{CDd}	16.65 ± 2.96^{Aa}	6.88 ± 1.30^{BCef}	9.74 ± 2.79^{Bc}	7.88 ± 0.80^{Bc}

Zeaxanthin	9.24 ± 1.22^{Bab}	15.50 ± 1.70^{Bab}	15.10 ± 0.81^{Ba}	30.73 ± 4.67^{Aa}	39.57 ± 5.32^{Aa}
9-*cis*-Antheraxanthin	1.56 ± 0.18^{Dd}	6.14 ± 0.83^{Bc}	4.49 ± 0.88^{BCf}	8.91 ± 1.68^{Ac}	3.57 ± 0.52^{CDc}
β-Carotene	6.21 ± 0.41^{Cc}	11.72 ± 0.60^{BCb}	8.98 ± 0.81^{BCde}	14.57 ± 1.28^{Bbc}	21.01 ± 3.87^{Ab}
Total	7.57 ± 0.28^{C}	14.44 ± 0.99^{B}	11.54 ± 0.75^{B}	18.22 ± 1.89^{B}	25.93 ± 4.35^{A}

FRESH, Fresh orange juice; UF-RT, ultrafrozen juice with thawing at room temperature; UF-FG, ultrafrozen juice with thawing in the fridge; UF-MW, ultrafrozen juice with thawing in the microwave oven; PAST, pasteurized juice. Values are the mean ± SD of 5 independent measures. Values within a column and within a row with different lowercase and uppercase letters respectively indicate statistically significant differences ($P < 0.05$).

Table 5. Bioaccessible carotenoid content (mg/250 mL) of Pinalate fresh orange juice and Pinalate orange juices subjected to different thermal treatments.

	Juice samples			
	UF-RT	UF-FG	UF-MW	PAST
Phytoene	28.22 ± 3.60	40.53 ± 4.33	43.32 ± 5.97	67.85 ± 7.25
Phytofluene	31.22 ± 4.00	40.23 ± 4.45	42.58 ± 5.57	67.57 ± 5.95
ζ-Carotene	33.27 ± 4.32	40.59 ± 4.31	41.38 ± 5.82	66.21 ± 5.74
9-*cis*-Violaxanthin	85.71 ± 4.35	75.50 ± 7.37	68.33 ± 3.82	62.51 ± 4.38

Zeaxanthin	37.80 ± 5.46	49.35 ± 5.11	57.29 ± 0.47	58.46 ± 2.97
9-*cis*-Antheraxanthin	54.95 ± 5.63	55.52 ± 2.39	59.12 ± 1.60	55.98 ± 5.37
β-Carotene	22.01 ± 4.48	28.70 ± 3.30	31.02 ± 7.49	63.70 ± 4.62
Total	32.18 ± 4.12	40.67 ± 4.22	42.18 ± 5.76	66.74 ± 6.03

FRESH, Fresh orange juice; UF-RT, ultrafrozen juice with thawing at room temperature; UF-FG, ultrafrozen juice with thawing in the fridge; UF-MW, ultrafrozen juice with thawing in the microwave oven; PAST, pasteurized juice. Values are the mean ± SD of 5 independent measures. Values within a column and within a row with different lowercase and uppercase letters respectively indicate statistically significant differences ($P < 0.05$).

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Supplementary Table 1. Degradation in percentage of carotenoid in Pinalate orange juices subjected to different thermal treatments.

	Juice samples			
	UF-RT	UF-FG	UF-MW	PAST
Phytoene	28.22 ± 3.60	40.53 ± 4.33	43.32 ± 5.97	67.85 ± 7.25
Phytofluene	31.22 ± 4.00	40.23 ± 4.45	42.58 ± 5.57	67.57 ± 5.95
ζ-Carotene	33.27 ± 4.32	40.59 ± 4.31	41.38 ± 5.82	66.21 ± 5.74
9-*cis*-Violaxanthin	85.71 ± 4.35	75.50 ± 7.37	68.33 ± 3.82	62.51 ± 4.38
Zeaxanthin	37.80 ± 5.46	49.35 ± 5.11	57.29 ± 0.47	58.46 ± 2.97
9-*cis*-Antheraxanthin	54.95 ± 5.63	55.52 ± 2.39	59.12 ± 1.60	55.98 ± 5.37
β-Carotene	22.01 ± 4.48	28.70 ± 3.30	31.02 ± 7.49	63.70 ± 4.62
Total or mean?	32.18 ± 4.12	40.67 ± 4.22	42.18 ± 5.76	66.74 ± 6.03

FRESH, Fresh orange juice; UF-RT, ultrafrozen juice with thawing at room temperature; UF-FG, ultrafrozen juice with thawing in the fridge; UF-MW; ultrafrozen juice with thawing with the microwave; PAST, pasteurized juice. Values are the mean ± SD of 3 independent measures.

Supplementary Table 2. Retention times in minutes and absorption maxima in nm of some compounds of the Pinalate orange juices.

Compound	Retention time	Absorption maxima
Compound 1	21.87	406, 430, 455
Compound 2	22.65	416, 439, 467
Compound 3	23.00	417, 440, 469
Compound 4	23.16	440, 463, 491

Supplementary Table 3. Increase in percentage of the carotenoid bioaccessibility in Pinalate orange juices subjected to different thermal treatments.

	Juice samples			
	UF-RT	UF-FG	UF-MW	PAST
Phytoene	66.28	38.21	109.65	211.60
Phytofluene	89.95	53.22	139.05	244.07
ζ-Carotene	99.55	58.48	155.80	259.52
9-*cis*-Violaxanthin	512.13	152.94	258.09	189.71
Zeaxanthin	67.75	63.42	232.58	328.25
9-*cis*-Antheraxanthin	293.59	187.82	471.15	128.85
β-Carotene	88.73	44.61	134.62	238.33
Total	90.75	52.44	140.69	242.54

FRESH, Fresh orange juice; UF-RT, ultrafrozen juice with thawing at room temperature; UF-FG, ultrafrozen juice with thawing in the fridge; UF-MW, ultrafrozen juice with thawing with the microwave; PAST, pasteurized juice. Values are the mean ± SD of 5 independent measures. To calculated the increase of the bioaccessibility the corresponding bioaccessibility found in the FRESH was taken as control.

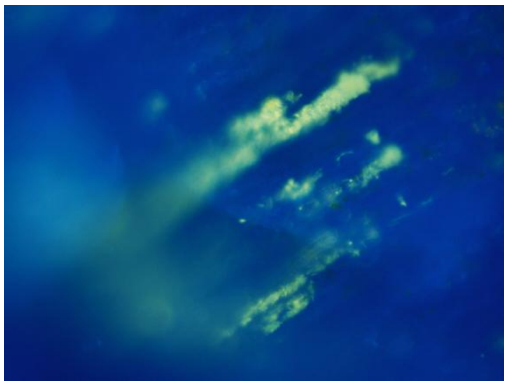
FIGURE CAPTIONS

Figure 1. **Representative micrographs of Pinalate orange pulp (A) and juice (B) and Navelate pulp (C) and juice (D) obtained by epifluorescence microscopy.**

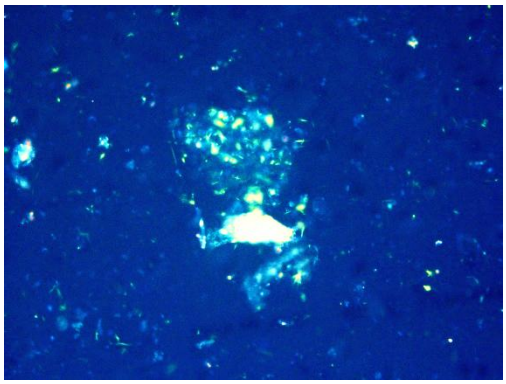
Figure 2. Representative micrographs of Pinalate orange pulp and juices obtained by Transmission Electron Microscopy. (A) Pulp (bar 1 µm); (B) Fresh orange juice (FRESH) (bar 1 µm); (C) Ultrafrozen juice with thawing in the fridge (bar 0.5 µm); (D) Ultrafrozen juice with thawing at room temperature (UF-RT) (bar 1 µm); (E) Ultrafrozen juice with thawing with the microwave oven (UF-MW) (bar 1 µm); (F) Pasteurized juice (PAST) (bar 0.5 µm). CW, cell wall; Mm, achlorophyllous membranes; Mt, mitochondria; Pg, plastoglobuli; s, starch grains; V, vesicles.

Figure 3. Particle size distribution of Pinalate fresh orange juice and Pinalate orange juices subjected to different thermal treatments.

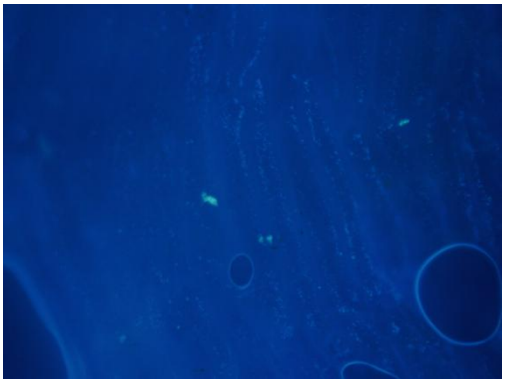
A (Pinalate pulp)



B (Pinalate juice)



C (Navelate pulp)



D (Navelate juice)

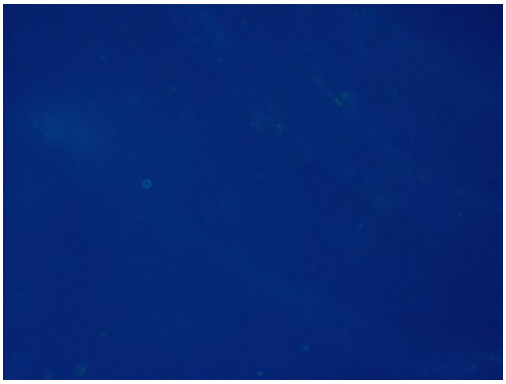


Figure 1

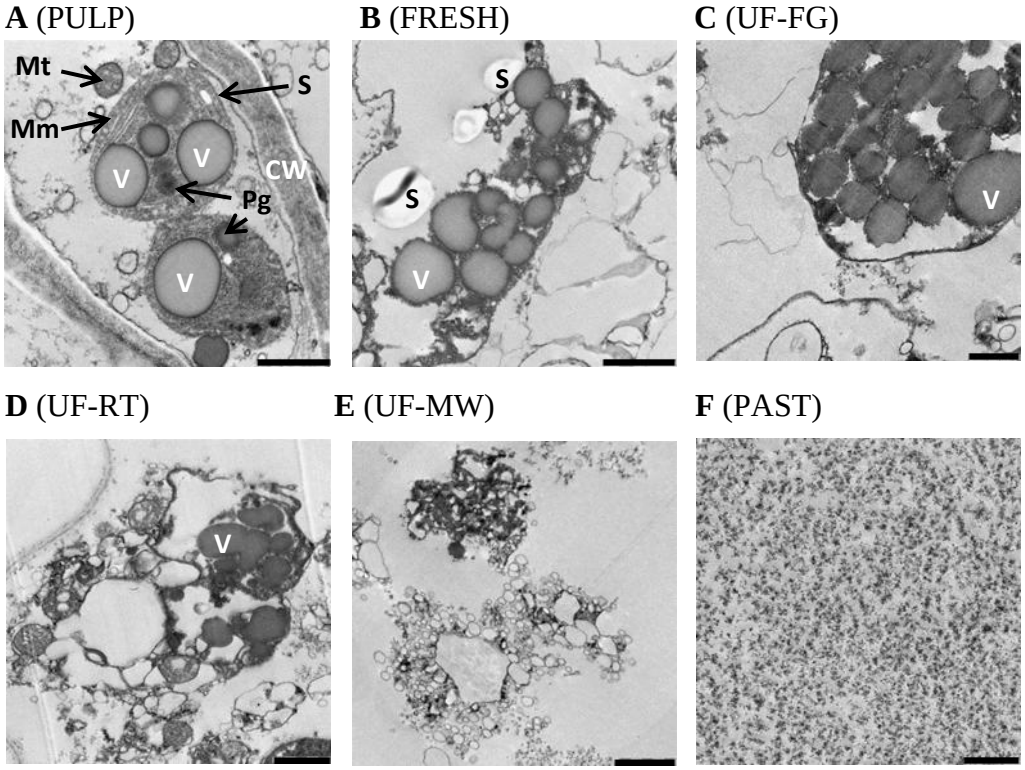


Figure 2

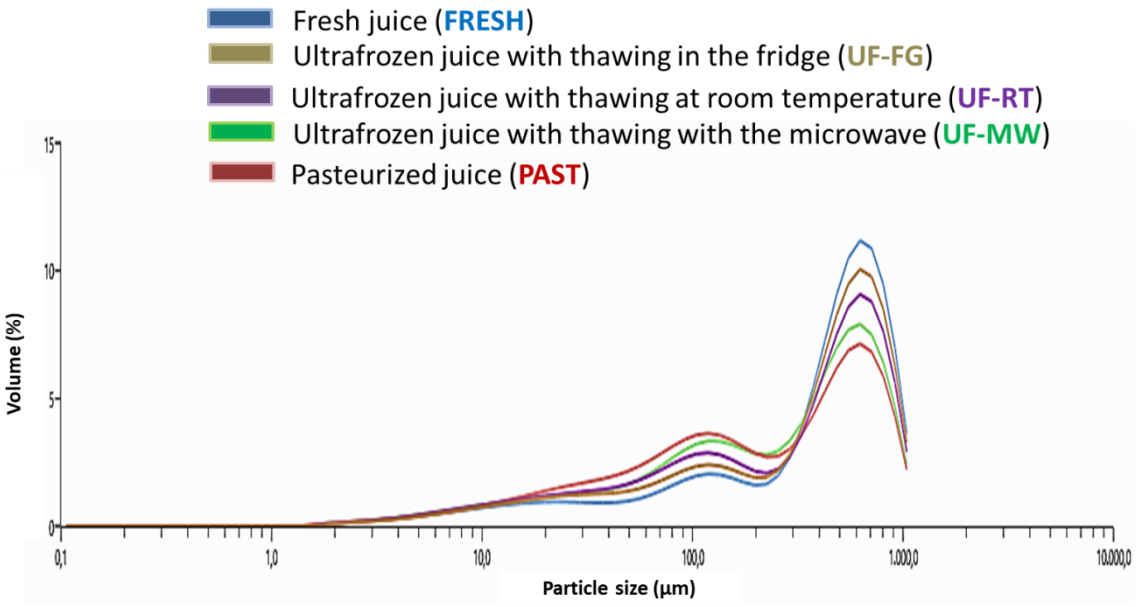

Fresh juice (FRESH)
Ultrafrozen juice with thawing in the fridge (UF-FG)
Ultrafrozen juice with thawing at room temperature (UF-RT)
Ultrafrozen juice with thawing with the microwave (UF-MW)
Pasteurized juice (PAST)
Volume (%)
15
10
5
0
0,1
1,0
10,0
100,0
1.000,0
10.000,0
Particle size (µm)

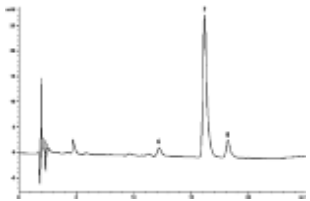
Figure 3

Graphical abstract

PINEAPPLE ORANGE JUICE SAMPLES

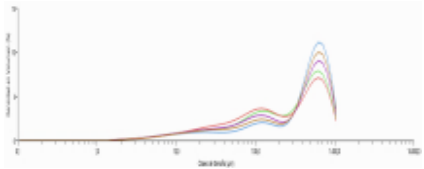
1. Fresh juice (**FRESH**)
2. Ultrafrozen juices thawed at room temperature (**RT**)
3. Ultrafrozen juices thawed in microwave oven (**MW**)
4. Ultrafrozen juices thawed in the fridge (**FG**)
5. Pasteurized juice (**PAST**)

Carotenoid analysis (HPLC)

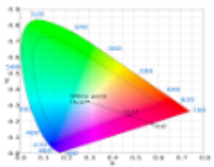


- **PHYTOENE (PT)**
- **PHYTOFLUENE (PTF)**
- ζ-Carotene
- β-Carotene
- Violaxanthin
- Antheraxanthin
- Zeaxanthin

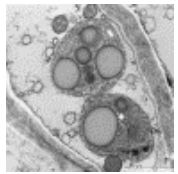
Particle Size Distribution (PSD)



Colour analysis



TEM images



BIOACCESSIBILITY:

PT > PTF > ζ-Carotene

CAROTENOID DEGRADATION:

Pasteurized juices: PT and PTF > Xanthophylls

Ultrafrozen juices: PT and PTF < Xanthophylls

PSD:

FRESH > FG > RT > MW > PAST

DEGRADATION OF SUBSTRUCTURES (TEM):

FRESH < FG < RT < MW < PAST

BIOACCESSIBILITY:

FRESH < FG < RT < MW < PAST