Bioaccessibility and antioxidant activity of Carotenoids in Ultrafrozen Orange Juices: Influence of Thawing Conditions.

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Abstract

Ultrafrozen orange juices (UFOJ) are produced to preserve the nutritional and sensory quality of the fresh juices. However the effects of the thawing conditions on the nutritional quality have been scarcely studied. To gain insight into this subject we have assessed the impact of different thawing conditions (microwave, room and refrigeration temperature) on the carotenoids levels and bioaccessibility in UFOJ. Other related properties, such as colour, and antioxidant activity were also evaluated. The results demonstrated that the bioactive carotenoid content and the antioxidant activity were significantly affected by the microwave thawing. However, these juices showed the highest values for the % of relative bioaccessibility of provitamin A carotenoids, when compared to the other thawing conditions. On the other hand, thawing at room or refrigeration temperatures did not have a negative impact neither on the colour, provitamin A and macular carotenoid compounds nor in the antioxidant activity.

KEYWORDS: antioxidant activity, bioaccessibility, carotenoids, frozen orange juices, thawing conditions

Abbreviations: orange juices (OJ); fresh orange juice (FOJ); room temperature (A), refrigeration temperature (R) and microwave (Mw), retinol activity equivalent (RAE); Trolox Equivalent Antioxidant Capacity Assay (TEAC).

1. Introduction

Orange juice (OJ) is one of the juices most widely consumed worldwide. It is a good source of nutritionally important compounds such as carotenoids, vitamin C and flavonoids. The carotenoid profile of orange juices is one of the most intricate among fruits (Dugo et al., 2008; Meléndez-Martínez, Britton, Vicario, & Heredia, 2008). These compounds have been associated with a lower risk of degenerative diseases in humans. The health effects of carotenoids depend on the amount consumed and on their bioavailability, which can be estimated as bioaccessibility using in vitro digestions. A number of factors that affect the bioaccessibility/bioavailability are described in the literature, as the nature and contents of carotenoids, presence of fat and fiber, food matrix, nutrient status, genetics, and interactions among these variables, as well as the effect of the industrial processes (Ornelas-Paz, Failla, Yahia, & Gardea-Bejar, 2008).

To preserve the nutritional and sensory quality of the fresh OJ several preserving methods are used in the industry. Among the different kinds of commercial orange juices those undergoing pasteurization treatments and those obtained from concentrate are the most popular. In both cases, the juices are submitted to thermal treatments that elongate their shelf life but can affect negatively its vitamin activity, its flavour, aroma, and colour (Lessin, Catignani, & Schwartz, 1997; Farnworth, Lagacé, Couture, Yaylayan, & Stewart, 2001; Lee & Coates, 2003). Due to the growing demand of consumers for healthier and natural foods, new alternative technologies that meet the primary objective of ensuring the conservation of the product have been assayed such as high pressure (HPP) (Polydera, Stoforos, & Taoukisa, 2005; Polydera, Stoforos, & Taoukis, 2003), pulsed electric fields (PEF) (Cortés, Esteve, & Frígola, 2008; Cortés, Esteve, Rodrigo, Torregrosa, & Frígola, 2006), microwave energy (Cinquanta, Albanese, Cuccurullo, & Di Matteo, 2010; Villamiel, Castillo, San Martín, & Corzo, 1998) or ultrasound (Tiwari, Muthukumarappan, ÖDonnell, & Cullen, 2008), although the scaling-up of some of them at an industrial levels is not profitable. Freezing quickly at very low temperatures ensures the nutritional quality, as well as the flavour and texture of foods, due to formation of small and uniform ice crystals. Ultra-high freezing rates can be achieved using liquid nitrogen. UFOJ,

from the Valencia late variety, has deeper and more appealing colouration due to of its higher content of carotenoid pigments, which is related to a higher quality (Meléndez-Martínez, Vicario, & Heredia, 2007; Meléndez-Martínez, Britton, Vicario, & Heredia, 2005). The amount of carotenoids is not affected by freezing, particularly quick freezing. However deteriorative process occurs, although at a very low rate, during storage (Thane & Reddy, 1997). Loss of nutritional value due to the freezing process has been extensively studied in fruits and vegetables, particularly in relation to vitamin C. According to Lee and Coates (1999) after 24 months of storage at -23 ºC, frozen and un-pasteurized orange juice reduced 19.2% its vitamin C content.

Thawing is a complex process, involving heat transfer and possibilities of a series of physical and chemical changes which may greatly affect the quality of the product. Common thawing conditions are room temperature (A), refrigeration temperature (R) or microwave (Mw) thawing. The first method (A) allows a rapid thawing, but there is a risk of potential growth of pathogens if food temperatures rise into the danger zone. In the second method (R) the temperature of the food remains 'safe' so there is very little pathogen build-up. Disadvantages of this method are longer thawing times and the requirement of space in refrigerators. The last method (Mw) may be the fastest although localized overheating should be avoided as it can be detrimental, particularly because the product can suffer chemical deterioration. From this, it can be inferred that it is important to adjust the freezing–thawing variables in order to preserve and retain the nutritional and sensory quality of juices.

The aim of this work was to assess the impact of different thawing conditions (A, R, Mw) on the carotenoid content and bioavailability of UFOJ, other properties related to carotenoids such as antioxidant activity and colour were also evaluated.

2. **Materials and Methods**

2.1. Chemicals

Extraction solvents were analytical-grade methanol, acetone and dichloromethane from Carlo-Erba (Milan, Italy). Analytic solvents were HPLC-grade methanol and methyl-tert-butyl-ether

98 (MTBE) from Merck (Darmstadt, Germany). Purified water was obtained from a NANOpure
99 Dlamond (Barnsted Inc.). Mineral salts (KCl, NaCl), sodium bicarbonate, chlorhydric acid,
100 pepsin (porcine gastric mucosa), pancreatin (porcine pancreas), bile salt, β-carotene, β-
101 cryptoxantin and zeaxanthin were purchased from Sigma-Aldrich (Steinheim, Germany). Other
102 carotenoids standards were either isolated from appropriate sources or semisynthesized in
103 accordance to standard procedures as explained elsewhere (Meléndez-Martínez et al., 2007).
104
105 *2.2. Samples*
106 Orange juice samples were taken directly from the commercial production line at the firm
107 "Zumos Pascual" (Palma del Rio, Cordoba, Spain) at different dates during the 2009 season
108 (from May to August). Valencia late oranges in an appropriate stage of maturity, corresponding
109 to a soluble solid content of 11-13 ºBrix, were mechanically extracted with an FMC© in line
110 Premium Juice Extractor (FMC Food Tech Citrus System, Lakeland, USA). The fresh industrial
111 squeezed OJ (F) samples were taken at this stage (six in total) and consisted of about 2 liters.
112 Ultrafreezing was carried in out by direct immersion in liquid nitrogen and posterior storage at -
113 20ºC until analysis.
114 The thawing conditions used were: room temperature (A), refrigeration temperature (R) and
115 microwave defrosting at maximum power (800w), 20 s (Mw). These conditions were selected
116 based on preliminary studies, in order to avoid overheating of the juice.
117 *2.3. Colour Measurement.*
118 The reflectance spectra were obtained by means of a CAS 140 B spectroradiometer (Instrument
119 Systems, Germany) fitted with a Top 100 telescope optical probe, a Tamron zoom mod. SP 23A
120 (Tamron USA, Inc., Commack, NY), and an external light source a white light 150W-metal-
121 halide lamp Phillips MHN-TD Pro (12900 lumen, 4200 K colour temperature) as source of
122 illumination. Blank measurements were made with distilled water against a white background.
123 The entire visible spectrum (380-770 nm) was recorded with a bandwidth of 1 nm, and the
124 Illuminant D65 and the 10º Observer were taken as references (CIE, 1978). The colour

parameters of the uniform colour space CIELAB L*; a*; b*; C^*_{ab} and h_{ab} were obtained directly from the apparatus. The colour data obtained were averages of three measurements. The colour differences (ΔE^*_{ab}) between two points in the CIELAB space are defined as the Euclidean distance between their locations in the three-dimensional space defined by L*, a*, and b*. This was calculating using the formula:

$$\Delta E^*_{ab} = \sqrt{\left(\Delta L^*\right)^2 + \left(\Delta a^*\right)^2 + \left(\Delta b^*\right)^2} \qquad (1)$$

where ΔL*, Δa* and Δb* are differences between the orange juice colour of fresh and pasteurized juice , and orange juice squeezed manual and squeezed industrial.

2.4. Titratable acidity and pH compound analysis

The titratable acidity expressed as citric acid was assessed by standard procedures. pH was measured with a GLP-21 GRINSON pHmeter. All analyses were made in triplicate.

2.5. Assessment of the in vitro antioxidant activity of lipophilic extracts by the TEAC method (Trolox Equivalent Antioxidant Capacity Assay).

The antioxidant activity of the lipophilic fraction of the OJs was assessed on aliquots of the same extracts used to determine carotenoids by HPLC.

The method used was based on the capture of the radical cation $ABTS^{\cdot+}$, generated in the reaction medium, compared to an antioxidant which produces the fall in absorbance at 734 nm (Re et al., 1999). The method measures the difference between the initial and the final absorbance due to the reduction experienced by the compound and can be used to assess the antioxidant capacity of the reducing substance.

The 2,2'-Azinobis(3-ethylbenzothiazoline- 6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) was produced by reacting a 7 mM ABTS aqueous solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{+}$ solution was diluted with ethanol to an absorbance of 0.7 at 734 nm

(30º). Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) was used as a standard for comparison for determination of the capacity of free radical scavenging. One mL of the ABTS radical solution was added to the cuvette and the absorbance was measured at time 0. Subsequently, 5μL and 15 μL of the same extracts used to determine carotenoids by HPLC were added to the cuvette. It stirred and incubated at 30°C. After 6 minutes, the absorbance was measured at 734 nm on a HP-8453 spectrophotometer equipped with temperature controller. The dose-response curve for Trolox consisted of plotting the absorbance at 734 nm as a percentage of the absorbance of the uninhibited radical cation (blank) and was based on triplicate determinations. The Trolox equivalent antioxidant activity (TEAC) was calculated by dividing the gradient of the curve of the sample and the gradient of the standard Trolox curve, taking into account the dilution used. The antioxidant activity of the lipophilic fraction was expressed in millimols of Trolox per L of orange juice.

2.6. In vitro digestion and bioaccessibility

The *in vitro* gastrointestinal digestion protocol used in this study was a combination of the methods proposed by Garret, Failla and Sarma (1999) and Liu, Glahn and Liu (2004) with slight modifications (Stinco et al., 2012). Briefly the method consisted of a pepsin-HCl digestion for 1 h (to simulate gastric digestion) and a pancreatic digestion with bile salts for 2 h at 37°C (to simulate small intestine digestion). For the gastric digestion, 1 mL of OJ was added to 1.8 mL of saline solution (140mM NaCl / 5 mM KCl) and 0.2 mL of pepsin solution (160 mg pepsin in 4 mL 0.1M HCl), the pH was adjusted to 2 by addition of HCl 0.1 M, and it was incubated in a shaker Max Q5000 (Thermo Fisher Scientific Inc., Waltham, MA) at 95 rpm and 37 °C for 1 h. For the pancreatic digestion, the pH of the partially digested mixture was raised to 6.9 by adding 0.1M $NaHCO_3$, followed by the addition of 0.25 mL of a mixture of bile extract and pancreatin (containing 2 mg/mL pancreatin and 12 mg/mL bile extract in 5 ml of 0.1 M $NaHCO_3$ solution). Samples were incubated in a shaker Max Q5000 (Thermo Fisher Scientific Inc., Waltham, MA) (95 rpm, at 37 °C) for 2 h to complete the intestinal phase. Transfer from the digesta to the aqueous-micellar phase was estimated by calculating the proportion of carotenoids in the

178 supernatants after low speed centrifugation (5000 x *g* for 20 min). The supernatants were used
179 for carotenoids analysis. The "relative bioaccessibility" was estimated by considering the
180 carotenoid content in the supernatant of the digest. The percentage of "relative bioaccessibility"
181 for each carotenoid was calculated as follows:

182 $$\%\ Bioaccessibility_{carotenoid} = \frac{mg/L_{carotenoid\_digest}}{mg/L_{carotenoid\_sample}} x100 \quad (2)$$

183

184 *2.7. Extraction and saponification of carotenoids*

185 The extraction and saponification of carotenoids were carried out according the method
186 described by Stinco et al. (2012). The extracting solvent was
187 dichloromethane/methanol/acetone, 50:25:25 v/v/v and saponification conditions were:
188 methanolic KOH (30% w/v) for 1 h under dim light and at room temperature, after which they
189 were washed with water to remove any trace of base. The coloured dichloromethane extracts
190 obtained from both OJs and supernatants were concentrated to dryness in a rotary evaporator at
191 temperature below 30 ºC and dissolved in 60 μL of ethyl acetate prior to their injection in the
192 HPLC system. The analyses were performed in triplicate.

193 *2.8. HPLC Analysis of Carotenoids*

194 The analyses of carotenoids were carried out according the method described by Stinco et al.
195 (2012). The identification of carotenoids was made by comparison of their chromatographic and
196 UV/vis spectroscopic characteristics with those of standards either isolated from appropriate
197 sources or semisynthesized in accordance to standard procedures as explained elsewhere
198 (Meléndez-Martínez et al., 2007). The carotenoid content of orange juice was worked out by
199 external calibration performed in compliance with recommended guidelines (Rodriguez-Amaya,
200 2001) from calibration curves constructed with the corresponding standards, as explained
201 elsewhere (Meléndez-Martínez et al., 2007). The total content was assessed as the sum of the
202 content of individual pigments. The analyses were performed in triplicate.

The vitamin A activity of the OJ samples was expressed in terms of retinol activity equivalents (RAE) (Food and Nutrition Board, 2002). The following formula was used for obtaining the RAE value and the results were referred to 1 L of OJ:

$$RAE = \frac{\mu g\ \beta\ carotene}{12} + \frac{\mu g\ \beta\ cryptoxanthin + \mu g\ \alpha\ carotene}{24} \quad (3)$$

2.9. Statistical Analysis.

Results were given as mean and standard deviation of independent determinations. Significant differences between the results were calculated by analyses of the variance (ANOVA). Statistically significant differences *($p<0.05$)* were determined using the Turkey multiple comparison procedure. Pattern recognition techniques, such as stepwise Linear Discriminant Analysis (SLDA), were applied on experiment standardized data to distinguish between different types of orange juices. All the statistical analyses were performed with Statistica v.8.0 software (StatSoft, 2007).

3. Results and Discussion

3.1. Carotenoids, antioxidant activity and Colour

Table 1 shows the carotenoid levels of the different OJ, and their vitamin activity expressed as retinol activity equivalents (RAE). Mw thawing decreased significantly *($p>0.05$)* the concentration of total carotenoids in relation to the untreated orange juice (FOJ) by 24%. The other thawing methods did not affect the total content in comparison to the control. Fratianni et al., (2010) studied the degradation of carotenoids in orange juice during microwave heating at different time/temperature conditions and also reported a decrease by 10-50% in Mw heated juices *($p<0.05$)* depending on the heating temperature and time (1 min, 70 ºC; 1 min, 75 ºC;1 min, 85 ºC) respectively.

Considering provitamin A carotenoids, the levels of β-cryptoxanthin, α-carotene and β-carotene, were significantly lower *($p <0.05$)* in MwOJ with respect to AOJ and ROJ and FOJ.

229 The greatest reduction (by 64-66%) was observed in the case of β-carotene. Similarly, Fratianni
230 et at. (2010) reported that β-carotene decreased *(p<0.05)* between 44 % - 65% when OJ heated
231 by Mw 1 min at 75ºC and 85ºC respectively. Consequently, the vitamin A activity of the
232 samples (RAE /L) was also reduced (50%). Other bioactive carotenoid as lutein, decreased by
233 22%, 22% and 25% compared to AOJ, ROJ and FOJ, respectively *(p<0.05)*. Similarly the
234 zeaxanthin levels were significantly lower than in AOJ, ROJ and FOJ (39-41%). This reduction
235 was higher than that previously reported by Cinquanta et al. (2010). Our results seem to indicate
236 that the Mw thawing reduced the nutritional value of OJs, due to a significant decreased
237 *(p<0.05)* of both provitamin A and macular carotenoids (lutein and zeaxanthin) in comparison
238 to the FOJ and even to the other thawing conditions. However the rest of carotenoids were not
239 affected. The concentration of 5,6 epoxides (violaxanthin, anteraxanthin and their isomers) and
240 5,8 epoxides (luteoxanthin and mutatoxanthin) were not affected by the thawing treatment, only
241 luteoxanthin + z-antheranxanthin levels decreased significantly *(p<0.05)* after Mw thawing
242 compared to the untreated samples (**Table 1**).
243 The antioxidant capacities of the lipophilic fraction of the orange juices were measured as free
244 radical-scavenging capacity by the TEAC method. Our results showed that, unlike the AOJ and
245 ROJ samples, the Mw-thawed juices showed a significantly lower antioxidant activity than the
246 controls (**Table 1**). This reduction was consistent with the results discussed above in relation to
247 a reduction on the carotenoid levels after the Mw-thawing.
248 The colourimetric and physicochemical parameters of the samples studied are summarized in
249 Table 2**.** According to the a*b* diagram the samples were located in the first quadrant, both a *
250 as b * took positive values **(Figure 1a)**. In relation to the colourimetric parameters, there were
251 no significant changes in the values of L*, a*, b*, C^*_{ab} and h_{ab} in the orange juices thawed at
252 room (AOJ) and refrigeration (ROJ) temperatures. However, the chroma (C^*_{ab}), b* and
253 lightness (L*) values were significantly higher (**Table 1**) in samples thawed by Mw relative to
254 the other thawing conditions. Thus the Mw juices appeared more vivid and lighter. These data
255 seem to indicate that thawing by Mw had a significant effect *(p<0.05)* on some colour attributes

of the juice when compared to other thawing conditions. A similar observation was reported by Cinquanta et al. (2010), who reported a significant increase in chroma when the juices underwent Mw heating at 75ºC for 2 min and at 85ºC for 1 min. Total colour difference (ΔE^*_{ab}) values between the control (FOJ) and the AOJ, ROJ, MwOJ samples were 1.91, 2.21 and 3.01 CIELAB units, respectively. The Mw treatment resulted in a higher colour difference *($p<0.05$)* visually appreciable, since the mean value of (ΔE^*_{ab}) was similar to the visual discrimination threshold (ΔE^*_{ab} >3). This result is in agreement with others presented in the literature (Cinquanta et al., 2010) that reported ΔE^*_{ab} increase of 0.77 and 5.73 CIELAB units, after MW heating for 1 and 2.5 min at 70 ◦C.

Typical reflection spectra corresponding to samples thawed at the different conditions tested (A, R, Mw) and the fresh orange juices (FOJ) are shown in **Figure 1b**. The four spectra were virtually identical in the interval 380-525 nm, although from this wavelength upwards it was observed that the reflection of light was clearly higher in the MwOJ and the lowest corresponded to the fresh juice. This fact seems to indicate that the different thawing methods studied affected slightly the reflection spectrum of the juices, the effect being perceptible only at wavelengths greater than 550 nm. Since the OJ carotenoids do not absorb light in that interval, it is tempting to hypothesize that the changes in the reflection of light could be attributed to changes in the juice particles.

These results are consistent with the effect of the thawing conditions on the carotenoid levels reported in AOJ and ROJ in relation to FOJ, as discussed above. Considering the overall results it could be inferred that the freezing and posterior thawing at room and refrigeration temperatures did not affect the carotenoid content nor their associated nutritional value, which indicate that these treatments could be good alternatives for the preservation of the colour and the carotenoid contents of orange juices.

As stated before, the antioxidant capacities of the lipophilic fraction of the orange juices were measured as free radical-scavenging capacity by the TEAC method. Our results showed that, unlike the AOJ and ROJ samples, the Mw-thawed juices showed a significantly lower

283 antioxidant activity than the controls (**Table 2**). This reduction was consistent with the results
284 discussed above in relation to a reduction of the carotenoid levels after the Mw-thawing.
285 To find out if it was possible to discriminate among the different OJs, a SLDA was carried out.
286 Stepwise forward selection algorithm was used and the different thawing conditions were
287 considered. A mathematical model that selected 12 variables, namely, β-carotene,
288 mutatoxanthin, lutein, b*, a*, TEAC, zeaxanthin, α-carotene, L*, violanxathin, antheraxanthin
289 and luteoxanthin, was obtained. It classified correctly 100% of the cases *($p<0.001$).* The
290 location of the juice samples on the plane defined by the three corresponding canonical
291 functions are presented in **Figure 2**. As it can be observed the first discriminant function
292 separated the samples corresponding to FOJ and MwOJ from the rest. The first discriminant
293 function was mainly related to lutein, α-carotene, violaxanthin and a* with positive sign, and
294 with β-carotene, b*, TEAC, antheraxanthin, luteoxanthin and zeaxanthin with negative sign.
295 The second discriminant function was mainly related to the variables mutatoxanthin and
296 L*(positive sign).

297 *3.2. Bioaccessibility of carotenoids*

298 **Table 3** shows the mean values of the carotenoids detected in the digests of the four types of
299 OJs after the in vitro digestion. The different thawing methods tested did not affect significantly
300 the levels of total carotenoids present in the digests. However, it was observed that the bioactive
301 carotenoids (provitamin A and macular carotenoids) were significantly higher *($p <0.05$)* in the
302 digest of the MwOJ relative to the digest of the AOJ and ROJ. However, the levels of β-
303 carotene and α-carotene, and therefore the RAE values, were significantly lower *($p<0.05$)*
304 relative to the digest of the FOJ. This observation suggests that the defrosting under certain
305 circumstances could have a negative impact in the provitamin A carotenoids. Interestingly,
306 when the microwave treatment was applied, the concentration of some carotenoids increased
307 slightly, specifically, the levels of β-cryptoxanthin, β-carotene and the RAE values. This could
308 indicate that the Mw thawing of the juices could enhance the release of carotenoids.

In order to estimate the % of relative bioaccessibility of bioactive carotenoids, (lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene), we calculated the ratio between the mean levels of each carotenoid in the juices (**Table 1**) and after in vitro digestion process (**Table 3**). Results are shown in **Figure 3**. The % of bioaccessibility of carotenoids, excepting lutein, in the AOJ and ROJ samples were significantly lower *($p>0.05$)* in comparison to the FOJ. This is a striking result since food processing (mechanical homogenization, heat treatment, and so on) can disrupt the cell membrane, leading to the release of membrane-bound compounds though increasing the bioaccessibility (Lemmens, Van Buggenhout, Oey, Van Loey, & Hendrickx, 2009; Stinco et al., 2012; Van Buggenhout et al., 2010; Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010).

Faulks and Southon (2005) reported that following the release from the food matrix, the major limiting factor for the absorption of carotenoids is their solubilisation into the digest. The decrease in the solubility of the carotenoids may be related to the presence of the pectin methyl-esterase (PME). This enzyme was not inactivated as a result of the freezing process. Cameron, Niedz and Grohmann (1994) reported that heating at 80 ºC for 2 min was required to inactivate it. Other researchers suggest 88 ºC for 10-15 s (Kimball, 1999) or 85 ºC for 25 s (Bull et al., 2004). Since the juices analyzed were not subjected to any heat treatment, besides room or refrigeration thawing, the PME responsible for the pectin hydrolysis may have produced a loss of cloudiness, which could have affected the solubilisation of carotenoids and therefore their bioaccessibility.

Of all the three thawing conditions tested, Mw-thaw showed the highest values of % relative bioaccessibility of provitamin A carotenoids (α-carotene, β-carotene and β-cryptoxanthin) (**Figure 3**). Cinquanta et al. (2010) reported that the heat sensitive fraction of PME was inactivated after about 1 min at 70ºC of Mw heating. This effect could explain to a certain extent why the bioaccessibility did not change, since, unlike the other methods studied, the solubility of the carotenoids did not decrease due to PME inactivation.

4. Conclusion

According to our results the Mw thawing resulted in a significant decrease in the provitamin A (expressed as RAE) and macular (lutein and zeaxanthin) carotenoids levels, accompanied by a significant lower antioxidant activity relative to the control juices. However, neither the colour nor the epoxycarotenoid levels were significantly affected as a result of the treatment. Contrastingly, the thawing of the OJs at room or refrigeration temperatures did not have a negative impact on the colour, provitamin A and macular carotenoids compounds and their associated antioxidant activity expressed as TEAC. Interestingly, the % of bioaccessibility of the provitamin A and macular carotenoids in the Mw-thawed samples was not significantly different to the control sample, in contrast to the other types of thawing methods tested. More research is needed to gain further insight into the influence of different thawing methods in the parameters related to carotenoid release and bioaccessibility.

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Fratianni et al, 2010 and Cinquanta et al., 2009, these articles describe the effects of microwave heating (for pasteurization purposes) on orange juice carotenoids. Articles by Lemmens et al. (2010) and Stinco et al. (2012) give some clues on the relevance of the particle size in the bioaccesibility of carotenoids. Finally Melendez-Martínez et al (2007) is the first article in which ultrafrozen orange juices are characterized.

461 **Figure captions**

462 **Figure 1a.** Location of the orange juices analyzed (AOJ: Room temperature, ROJ: Refrigeration
463 temperature, MwOJ: Microwave treat and FOJ: Fresh orange juices) in the a*b* plane.

464 **Figure 1b.** Typical reflection spectra of Orange Juice samples (AOJ: Room temperature, ROJ:
465 Refrigeration temperature, MwOJ: Microwave treat and FOJ: Fresh orange juices)

466 **Figure 2.** Scatterplot of samples in the plane defined by the canonical functions when the
467 carotenoid contents are considered for discrimination between Fresh orange juices (FOJ) and
468 room temperature (AOJ), refrigeration temperature (ROJ), Microwave treat (MwOJ)

469 **Figure 3.** Relative bioaccessibility (%) of the bioactive carotenoids in the Orange Juice samples
470 (AOJ: Room temperature, ROJ: Refrigeration temperature, MwOJ: Microwave treat and FOJ:
471 Fresh orange juices)

472

Table 1. Carotenoids levels (mg/L) and Retinol Activity Equivalents (RAE) in the orange juices analyzed (orange juices thawed at Room temperature AOJ: Refrigeration temperature ROJ, Microwave MwOJ and Fresh orange juices FOJ)

Peak	Identification	AOJ	ROJ	MwOJ	FOJ
1	violaxanthin isomers	3.31 ± 0.97[a]	3.57± 0.80[a]	2.82 ± 0.51[a]	3.38 ± 0.91
2	luteoxanthin + (Z)-antheraxanthin	1.86 ± 0.57[a]	1.99 ± 0.45[a]	1.38 ± 0.32[a*]	1.98 ± 0.51[*]
3	9-cis-violaxanthin + antheraxanthin	6.90 ± 1.44[a]	7.15 ± 1.82[a]	5.90 ± 0.68[a]	7.09 ± 1.73
4	cis-luteoxanthin	0.51 ± 0.14[a]	0.49 ± 0.17[a]	0.42 ± 0.06[a]	0.51 ± 0.12
5	mutatoxanthin	1.05 ± 0.19[a]	1.04 ± 0.19[a]	0.87 ± 0.07[a]	0.87 ± 0.16
6	lutein	1.21 ± 0.16[a]	1.21 ± 0.15[a]	0.94 ± 0.10[b*]	1.25± 0.18[*]
7	mutatoxanthin	1.05 ± 0.23[a]	0.97± 0.26[a]	0.90 ± 0.16[a]	0.96± 0.27
8	zeaxanthin	2.28 ± 0.31[a]	2.31 ± 0.28[a]	1.36 ± 0.19 [b*]	2.23 ± 0.39[*]
9	antheraxanthin	3.23 ± 0.69[a]	3.35± 0.85[a]	2.62± 0.24[a]	3.27 ± 0.78
10	zeinoxanthin	0.62 ± 0.10[a]	0.62 ± 0.07[a]	0.42 ±0.02[b*]	0.61 ± 0.12[*]
11	β-cryptoxanthin	1.79 ± 0.38[a]	1.76 ± 0.21[a]	0.85 ± 0.12[b*]	1.76 ± 0.47[*]
12	(Z)-ζ-carotene isomer	not quantified	not quantified	not quantified	not quantified
13	α-carotene	0.30 ± 0.02[a]	0.30 ± 0.03[a]	0.22 ± 0.02[b*]	0.29 ± 0.03[*]
14	β–carotene	0.84 ± 0.09[a]	0.78 ± 0.13[a]	0.28 ± 0.06[b*]	0.80± 0.15[*]
	Total Carotenoids	24.96± 4.66[ab]	25.53± 5.03[a]	19.00 ± 1.86[b*]	24.99± 5.20[*]
	RAE	157.29 ± 21.53[a]	150.77 ± 17.64[a]	68.09± 9.85[b*]	152.36 ± 31.67[*]
	TEAC values	0.046±0.007[a]	0.047±0.005[a]	0.038±0.003[b*]	0.053±0.005[*]

[a,b,c] Different superscripts within the same row indicate statistically significant differences ($p < 0.05$) among different thawing methods (A,R, and Mw).

* Within the same row indicate statistically significant differences ($p < 0.05$) between frozen *vs* fresh juices (AOJ *vs* FOJ, ROJ *vs* FOJ and MwOJ *vs* FOJ)

Table 2. Physicochemical and colourimetric parameters for the different orange juices analyzed (orange juices thawed at Room temperature AOJ, Refrigeration temperature ROJ, Microwave MwOJ and Fresh orange juices FOJ)

Colourimetric parameters		**AOJ**	**ROJ**	**MwOJ**	**FOJ**
	L*	77.67 ± 0.34^{a}	78.82 ± 0.29^{a}	79.01 ± 0.20^{b}	77.93 ± 1.43
	a*	14.23 ± 1.06^{a}	14.38 ± 0.90^{a}	$15.00 \pm 0.82^{a*}$	$13.79 \pm 0.78^{*}$
	b*	72.36 ± 1.40^{a}	73.74 ± 1.56^{ab}	74.69 ± 1.30^{b}	73.36 ± 1.48
	C^*_{ab}	73.75 ± 1.53^{a}	75.13 ± 1.67^{ab}	76.19 ± 1.38^{b}	74.65 ± 1.55
	h_{ab}	78.88 ± 0.67^{a}	78.97 ± 0.52^{a}	$78.64 \pm 0.50^{a*}$	$79.36 \pm 0.47^{*}$
Physicochemical parameters					
	pH	3.61 ± 0.81^{a}	3.61 ± 0.10^{a}	3.72 ± 0.07^{a}	3.68 ± 0.10
	Acidity %[†]	0.79 ± 0.03^{a}	0.78 ± 0.03^{a}	0.82 ± 0.05^{a}	0.82 ± 0.04

[a,b,c] Different superscripts within the same row indicate statistically significant differences ($p < 0.05$) between different thawing methods (A,R, and Mw).

* Within the same row indicate statistically significant differences ($p < 0.05$) between frozen *vs* fresh juices (AOJ *vs* FOJ, ROJ *vs* FOJ and MwOJ *vs* FOJ)

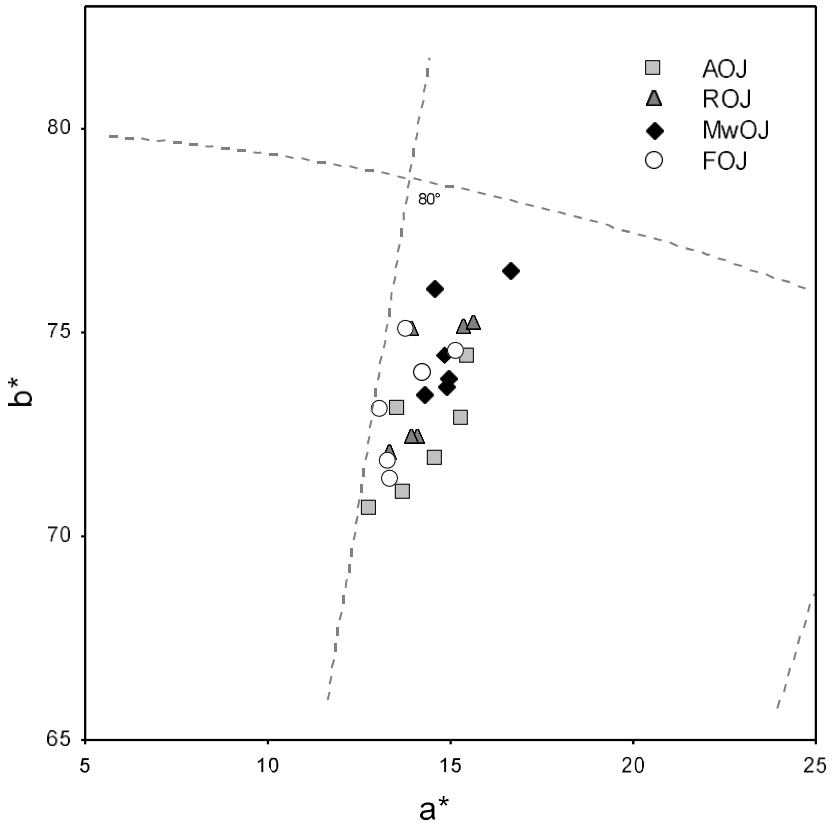
Table 3. Carotenoids levels in the digests (mg/L) and Retinol Activity Equivalents (RAE) in the orange juices analyzed (orange juices thawed at Room temperature AOJ, Refrigeration temperature ROJ, Microwave MwOJ and Fresh orange juices FOJ)

Peak	Identification	AOJ	ROJ	MwOJ	FOJ
1	violaxanthin isomers	1.19 ± 0.50^{a}	1.35 ± 0.35^{a}	1.37 ± 0.35^{a}	1.32 ± 0.41
2	luteoxanthin + (Z)-antheraxanthin	0.95 ± 0.44^{a}	1.10 ± 0.30^{a}	1.16 ± 0.34^{a}	1.04 ± 0.33
3	9-cis-violaxanthin + antheraxanthin	2.75 ± 1.08^{a}	2.90 ± 0.78^{a}	2.90 ± 0.64^{a}	2.80 ± 1.00
4	cis-luteoxanthin	0.38 ± 0.18^{a}	0.46 ± 0.14^{a}	0.56 ± 0.19^{a}	0.42 ± 0.15
5	mutatoxanthin	0.49 ± 0.16^{a*}	0.57 ± 0.08^{ab}	0.67 ± 0.09^{b}	0.83 ± 0.33*
6	lutein	0.49 ± 0.13^{a}	0.66 ± 0.10^{ab}	0.71 ± 0.09 ^{b}	0.57 ± 0.13
7	mutatoxanthin	0.54 ± 0.21^{a}	0.83 ± 0.58^{a}	0.64 ± 0.19^{a}	0.62 ± 0.22
8	zeaxanthin	0.66 ± 0.19^{a*}	0.93 ± 0.15^{b}	1.09 ± 0.11^{b}	1.09 ± 0.25*
9	antheraxanthin	1.40 ± 0.57^{a}	1.63 ± 0.49^{a}	1.75 ± 0.42 ^{a}	1.64 ± 0.57
10	zeinoxanthin	0.23 ± 0.06^{a*}	0.28 ± 0.04^{ab}	0.31 ±0.03^{b}	0.32 ± 0.07*
11	β-cryptoxanthin	0.48 ± 0.17^{a*}	0.67 ± 0.12^{ab*}	0.76 ± 0.10^{b}	0.94 ± 0.26*
12	(Z)-ζ-carotene isomer	not quantified	not quantified	not quantified	not quantified
13	α-carotene	0.11 ± 0.01^{a*}	0.12 ± 0.02^{ab*}	0.13 ± 0.01^{b*}	0.15 ± 0.02*
14	β–carotene	0.16 ± 0.05^{a*}	0.27 ± 0.07^{b*}	0.34 ± 0.04^{b}	0.42 ± 0.10*
	Total Carotenoids	9.83 ± 3.68^{a}	11.77 ± 2.47^{a}	12.41 ± 2.42^{a}	12.18 ± 3.42
	RAE	38.81 ± 11.14^{a*}	55.68 ± 10.20^{b*}	66.84 ± 9.87^{b}	80.43 ± 19.31*

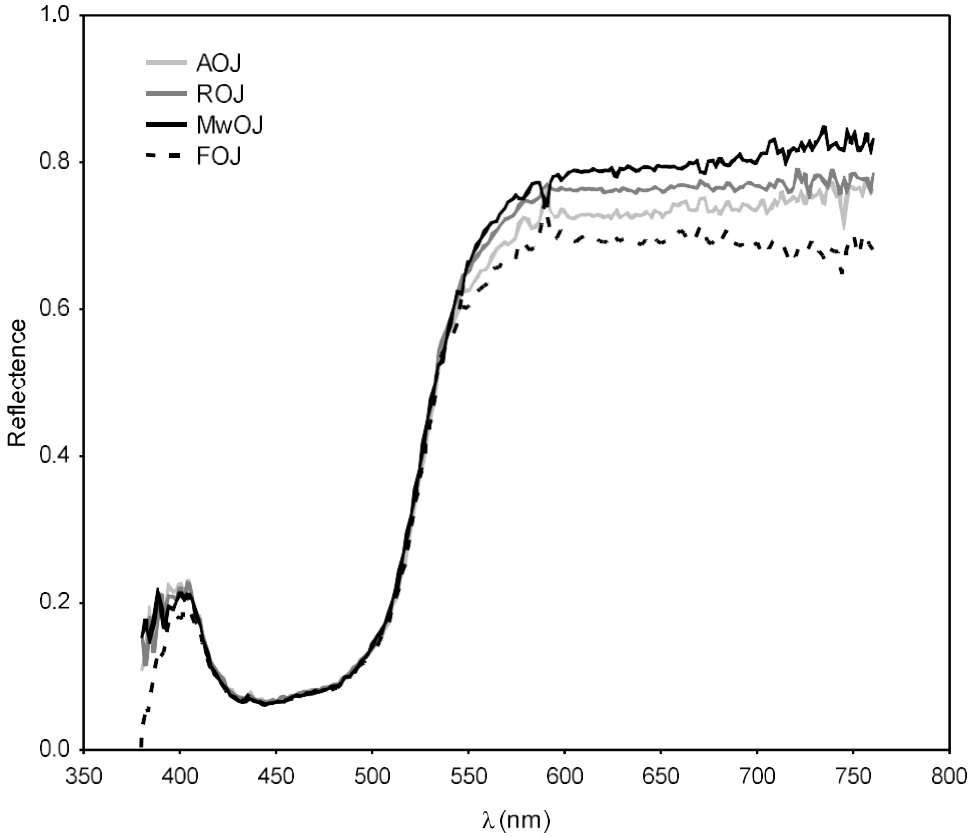
^{a,b,c} Different superscripts within the same row indicate statistically significant differences ($p < 0.05$) among different thawing methods (A,R, and Mw).

* Within the same row indicate statistically significant differences ($p < 0.05$) between frozen *vs* fresh juices (AOJ *vs* FOJ, ROJ *vs* FOJ and MwOJ *vs* FOJ)

1 **Figure 1**


AOJ
ROJ
MwOJ
FOJ
80°
b*
a*
80
75
70
65
5
10
15
20
25

(a)


AOJ
ROJ
MwOJ
FOJ
Reflectence
λ (nm)
1.0
0.8
0.6
0.4
0.2
0.0
350
400
450
500
550
600
650
700
750
800

(b)

Figure 2

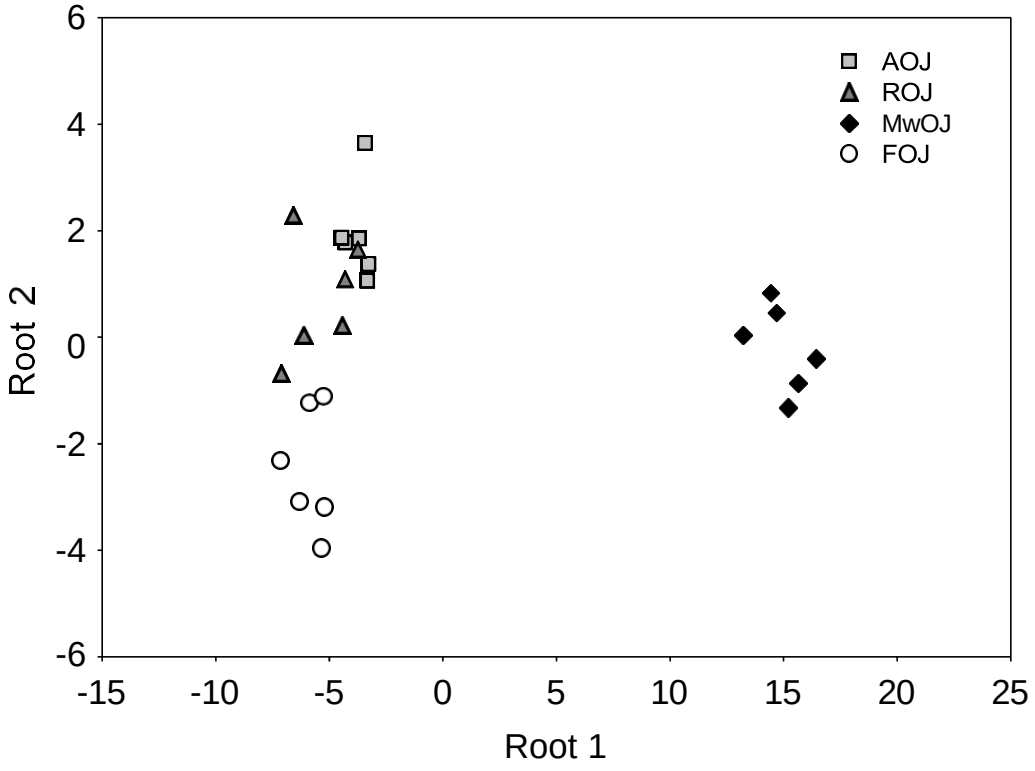
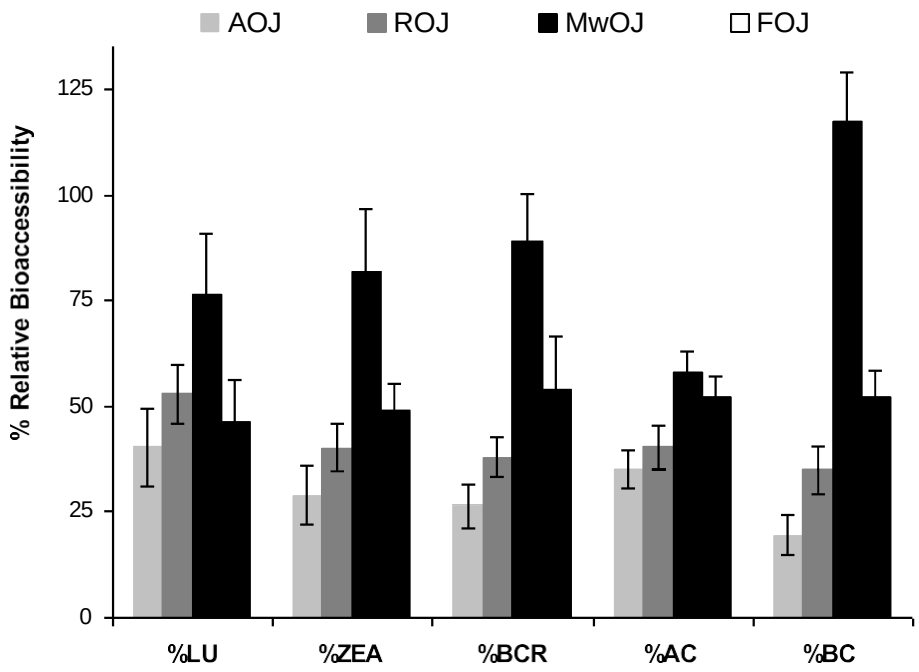


Figure 3


AOJ
ROJ
MwOJ
FOJ
% Relative Bioaccessibility
125
100
75
50
25
0
%LU
%ZEA
%BCR
%AC
%BC