**Effect of high temperature-conditioning treatments on quality, flavonoid
composition and vitamin C of cold stored ‘Fortune’ mandarins**

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ABSTRACT

The effect of high temperature-conditioning treatments (1-2 days at 37 °C) on fruit quality, flavonoids, total antioxidant capacity (TAC) and vitamin C was investigated in chilling-sensitive 'Fortune' mandarins subjected to single or double quarantine treatments (16 or 32 days at 1.5 °C, respectively). High temperature-conditioning treatments, which reduced chilling injury, allowed fruits to withstand quarantine treatments without affecting fruit quality, vitamin C or TAC. Hesperidin and isorhoifolin were the most abundant flavonoids followed by didymin and narirutin, whereas the polymethoxylated flavones (PMFs) nobiletin and tangeretin were the less abundants. Didymin and narirutin slightly increased (~1.5-fold) at 1.5 °C. A 4-fold increase occurred in eriocitrin, though its concentration was much lower. Small differences in flavonoids were found between non-conditioned fruit and fruit conditioned for 1 day after cold storage and their concentration in carpellary membranes were, in general, much higher than in juice. Therefore, fruit conditioning at 37 °C allows chilling-sensitive citrus cultivars to withstand quarantine treatments without having deleterious effects on fruit quality, vitamin C or relevant flavonoids.

Keywords: antioxidant capacity; Citrus fruit; chilling; cold quarantine storage; phenolics; carpellary membranes; high temperature-conditioning.

1. Introduction

Because of the beneficial effects of phenolics on several diseases, the interest in these compounds has been growing in the last years (Tripoli et al., 2007). Flavonoids are the most abundant phenolics in citrus fruits (Nogata et al., 2006). The highest concentrations in all species of the genus *Citrus* correspond to 7-*O*-glycosylflavanones, followed by flavones, flavonols and the fully polymethoxylated flavones (PMFs). Studies characterizing how phenolic composition is affected by fruit preharvest conditions or by industrial techniques have been performed in citrus fruits (Del Caro, Piga, Vacca, & Aggabio, 2004; Sentandreu, Navarro, & Sendra, 2007; Girennavar, Jayaprakasha, & Patil, 2008). However, few studies have addressed the influence of postharvest treatments needed to maintain fresh fruit quality on citrus phenolics (Vanamala et al., 2007; Rapisarda, Bellomo, Fabroni, & Russo, 2008). Furthermore, these studies have been mainly performed in the peel or in citrus juices rather than in the edible portion (pulp) containing carpellary membranes, which may be partly removed during juice extraction.

Storage of fruits to near-freezing temperatures for at least 16 days is required in the quarantine security treatments against Mediterranean fruit fly (Medfly, *Ceratitis capitata*) for citrus fruit exportation. Fruits of many citrus cultivars are very sensitive to chilling injury (CI) and develop necrosis in the flavedo (outer part of the peel) at these temperatures. Therefore, fruit of chilling-sensitive citrus cultivars, as ‘Fortune’ mandarins (hybrid of ‘Dancy’ mandarin x ‘Clementine’ mandarin) cannot tolerate these treatments.

Although the tolerance of citrus fruits to chilling may be increased by previous high-temperature conditioning treatments at temperatures ranging between 30 and 52 °C (Martínez-Téllez & Lafuente, 1997; Schirra, Mulas, Fadda, & Cauli, 2004; Lafuente & Zacarías, 2006), the influence of these treatments on nutritional quality of cold stored citrus fruit is little

known. Their effect on vitamin C content in the pulp of different citrus cultivars has been examined (Schirra, Mulas, Fadda, & Cauli, 2004; Pérez et al., 2005b). The effect of heating peel or juice from citrus fruit at near boiling temperatures (≥ 95 °C) has been also evaluated (Xu, Ye, Chen, & Liu, 2007; Igual, García-Martínez, Camacho, & Martínez-Navarrete, 2010). However, the influence of less extreme high temperatures that reduce CI (< 52 °C) on phenolic accumulation in the edible portion of chilling-sensitive citrus cultivars is almost unknown. To our knowledge, there is only one report examining changes in specific phenolics induced by a short (2 min) hot water dip treatment at 50 °C in the whole fruit of Kumquat stored at a non-chilling temperature (17 °C) and only two flavonoids were identified (Schirra et al., 2008). The effect of low temperature on anthocyanins and on total flavonoids, the major polyphenols in blond citrus cultivars, has been also examined (Rapisarda, Bellomo, Fabroni, & Russo, 2008), but no information exists about the effect of storing citrus fruits at low temperature on specific flavonoids.

The aim of this work was to evaluate the effect of high-temperature (37 °C) conditioning treatments on fruit quality of ‘Fortune’ mandarins subjected to cold quarantine treatments, with special attention to changes in flavonoids, vitamin C and total antioxidant capacity (TAC) in the edible portion. Furthermore we examined flavonoids both in the juice and in the carpellary membranes of the segments whose loss during juice extraction may lead to differences in nutritional value between juice and fresh fruit.

2. Materials and methods

2.1. Fruit samples and postharvest treatments

‘Fortune’ (*Citrus clementina* Hort. Ex Tanaka x *Citrus reticulata*, Blanco) mandarins were harvested in February when commercially mature at even intervals around the tree from

6 tress planted uniformly 4 x 5 m and grown at Valencia, Spain. Fruits were selected for homogeneous size (medium) and maturity stage, free from defects, and randomly divided in three batches, 210 fruits each, in order to test three different storage conditions. The first batch (control non-conditioned fruits) was immediately exposed to cold quarantine at 1.5 °C and 80-85% relative humidity (RH). Fruits of the second and third batch were subjected to hot air treatment at 37 °C for 1 (batch 2) or 2 (batch 3) days, respectively, in thermostatic chambers. The temperature and RH (90-95% RH) of the heated chamber were monitored simultaneously by electronic thermohygrograph. Following heat conditioning treatments, fruits of batches 2 and 3 were exposed to cold quarantine as the control fruits. Fruits from each batch were divided in two groups. The first group contained 30 fruits that were used to evaluate CI along fruit storage, while the second group contained 30 fruits per storage period to evaluate changes in fruit quality parameters, total antioxidant capacity (TAC), vitamin C and in the concentration of phenolic compounds. Non-conditioned and conditioned fruits were stored for 16 days at 1.5 °C, to simulate a single quarantine treatment, or for 32 days at the same temperature, as a double quarantine treatment may be required in some instances, and then transferred to 20 °C for 4 days to simulate a shelf life (SL) period.

2.2. *Quality analysis*

Changes in fruit quality were assessed by determining the effect of treatments on peel damage and on fruit colour, firmness and weight loss, and on pulp total acidity, total soluble solid content (TSSC) and maturity index as described by Holland, Sala, Menezes, and Lafuente (1999). Peel colour was analyzed by using a Minolta CR-300 Chromameter (Konica Minolta Inc, U.S.A.) with a measuring area of 8 mm at four locations around the equatorial plane of the fruit. The CIELAB L^* , a^* , b^* colour notation system was used and values are reported as a colour index (IC) ranging from -20 to 20, which was calculated according to the

formula $IC = (1000 \times a^*/(L^* \times b^*))$ (Pérez et al., 2005b). Values of $IC < 20$ = green, $IC 0$ = breaker, $IC > 20$ = orange colour. TSSC of the pulp was measured by using an Atago/X-100 digital refractometer (Atago Co. Ltd, Tokyo, Japan) and expressed as °Brix. Acids content was titrated with 0.1 N NaOH using phenolphthalein as indicator and expressed as percentage of citric acid and the maturity index calculated by dividing the °Brix by the acid content. Fruit firmness was determined by using a manual penetrometer (Magness-Taylor, Spain) equipped with a 7.5 mm diameter flat tip at three locations around the equatorial plane of the fruit and the data are expressed in Kg. A rating scale from 0 to 3, based on surface necrosis and intensity of browning, was used to evaluate the extent of damage and the average CI index calculated using the following formula: $CI\ index = \Sigma (CI\ scale\ (0-3) \times number\ of\ fruit\ in\ each\ class) / total\ number\ of\ fruit$ (Holland, Sala, Menezes, & Lafuente, 1999). CI was scored as 0, when fruits showed no CI symptoms; as 1 when CI covered less than 10% of the peel surface, as 2 when CI covered up to 30%, and as 3 when CI covered over the 30% of the peel surface. The results of each quality analysis are the mean of three replicate samples containing a pool of 10 fruits each, accounting for a total of 30 mandarins per sampling point.

2.3. Preparation of samples for phenolic analysis

Phenolic analysis was performed in the pulp (edible portion), containing both juice and carpellary membranes, and also separately in both pulp parts. Pulp samples were prepared after carefully removing the peel and part of the endocarp segments of each fruit were cut into smaller pieces, immediately frozen in liquid nitrogen, mixed, grounded and homogenized to a fine powder with a coffee mill and stored at -80 °C until further analysis. The remaining segments were used for preparation of juice and carpellary membrane samples as described above after separating them with a razor. Three biological replicates, each pooled by 10 fruits, were prepared for each analysis.

2.4. Phenolic analysis by HPLC

The analysis of phenolics was carried out by HPLC according to the method of Ballester, Izquierdo, Lafuente, and González-Candela (2010). Briefly, phenolic compounds were extracted twice from the previously homogenized pulp, juice and carpellary membrane frozen materials with 80% methanol by using a Mini Beadbeater 8 Cell Disruptor (Biospec Products, Inc.) and after centrifugation at $11600 \times g$ for 8 min the combined supernatants were filtered through $0.45 \mu\text{m}$ PTFE Millipore filters for HPLC analysis. To that end, a Waters liquid chromatography system (Waters, Barcelona, Spain) equipped with a quaternary pump (Waters 600) and photodiode array (Waters 2996) detector was used. Separation was performed at 35°C by using a Luna C18 (Phenomenex, Inc.) column ($250 \times 4.6 \text{ mm}$, $5\mu\text{m}$) coupled to a $\mu\text{Bondapak C18}$ guard column (Waters, Barcelona, Spain) ($10\mu\text{m}$) and a binary gradient of water adjusted to pH 2.5 with phosphoric acid (A) and acetonitrile (B) at a constant flow of 0.8 mL min^{-1} . The solvent composition changed in a linear gradient from 99% A and 1% B to 70% A and 30% B during 60 min. During the next 45 min, the solvent composition was changed to 1% A and 99% B, at which point it was held for 10 min. Phenolics were detected by setting the photodiode array detector (PDA) to scan from 200 to 400 nm throughout all the elution profile. Quantification of peak areas and identification of phenolic compounds, by comparison of the spectra and retention times (RT) with authentic standards, were achieved by using the Empower Software (Waters, Barcelona, Spain). For each elution a Maxplot chromatogram was obtained, which plots each peak at its corresponding maximum absorbance wavelength. Thus, phenolics peaks of samples and standards were integrated at their individual maximum wavelengths and their contents were calculated using the calibration curves of the corresponding standards. Duplicate samples, which were within the

linear range of the corresponding phenolic standard curves, were analyzed for each biological replicate and the results are the mean of three biological replicate samples.

2.5. Total antioxidant capacity and vitamin C

The ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)) free radical decolouration and DPPH· (1,1-diphenyl-2-picryl-hydrazyl) free radical-scavenging assays were used to evaluate antioxidant capacity. The free radical DDPH· assay was performed as described by Lo Scalzo et al. (2004) with minor modifications. The samples were extracted twice with 80% methanol and after centrifugation at $11600 \times g$ for 8 min the combined supernatants were filtered through 0.45 μm PTFE Millipore filters. The supernatants (200 μL at a final concentration of 100 mg FW sample mL^{-1}) were added to a cuvette containing 2.8 mL of 0.1 mM DPPH·. The control sample was prepared using 200 μL 80% methanol and 2.8 mL of 0.1 mM DPPH·. The optical density was determined at 517 nm at 25 °C and the radical scavenging activity (S) of each extract expressed by the formula $S = 100 - 100 \times (A_x/A_o)$ (Lo Scalzo et al., 2004). In this formula, A_x was the optical density of DPPH· in presence of the sample and A_o the optical density of DPPH· in the absence of the sample. The ABTS assay was performed as previously reported (Rice-Evans et al., 1995) using a standard curve constructed with different concentrations of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Briefly, the sample was extracted with 5 mM PBS (phosphate buffered saline) buffer (pH 7.4) and after a three fold dilution the extracts were centrifuged at $11600 \times g$ for 5 min at 4 °C to eliminate cell debris. TAC was measured using the ferrylmyoglobin/ABTS spectrophotometric assay generating the $\text{ABTS}^{\bullet+}$ radical cation from the interaction of metmyoglobin and hydrogen peroxide. By this method, 120 μl of the diluted extract at a final concentration of 100 mg FW sample mL^{-1} reacted for 6 min in a cuvette containing 2.20 mL of 5mM PBS, 90 μL 5mM ABTS, 60 μL of 140 μM metmyoglobin and

225 μ L of 1 mM hydrogen peroxide. Absorbance readings were taken immediately after adding the hydrogen peroxide and periodically at 734 nm for up to 6 min and the reaction was performed at 30 °C. All the process was done in absence of light. Vitamin C was determined by the official method (AOAC, 1990) as previously reported by Del Caro, Piga, Vacca, & Aggabio (2004) and Schirra, Mulas, Fadda, & Cauli (2004). All the analysis were performed in duplicate extracts from each biological sample and the results are the mean of three biological replicate samples.

2.6. Standards

Eriocitrin (eriodictyol-7-O-rutinoside), narirutin (naringenin-7-O-rutinoside), isorhoifolin (apigenin-7-O-rutinoside) and didymin (isosakuranetin-7-O-rutinoside), also known as neoponcirin, were purchased from Extrasynthèse (Extrasynthèse, Genay, France), rutin (quercetin-3-O-rutinoside) from Sigma (Sigma, Spain) and hesperidin (hesperetin-7-O-rutinoside) from Fluka (Fluka, Spain). The PMFs nobiletin (3',4',5,6,7,8-hexamethoxyflavone) and tangeretin (4',5,6,7,8-pentamethoxyflavone) were kindly supplied by Dr. J.M. Sendra (Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Valencia, Spain). Reagents for vitamin C, ABTS and DPPH $\cdot$ assays were purchased from Sigma.

2.7. Statistics

The values are the means of three replicate samples $\pm$ SEM. Data were evaluated using Statgraphics.5.1 Software (Manugistics, Inc.) and Tukey's test was performed to identify significant differences between samples at $p \leq 0.05$.

3. Results and discussion

3.1. Influence of high temperature-conditioning on quality of cold stored mandarins

CI symptoms were manifested in non-conditioned 'Fortune' mandarins as small brown pit-like depressions on the peel after 1 week of storage at 1.5 °C and continuously increased during cold storage. Fruit conditioning for 1 or 2 days at 37 °C was very effective reducing CI after the single and the double quarantine treatments as CI indexes of these fruits were very low and negligible from the commercial point of view, even after the SL period (Fig. 1).

Postharvest treatments may have beneficial effect on certain horticultural crops and detrimental on others (Mulas & Schirra, 2007; Alasalvar et al., 2010). The influence of heat conditioning on the quality of 'Fortune' mandarins exposed to the single and double quarantine treatments is shown in Table 1. A 2.6% weight loss occurred by 2 days in fruit held at 37 °C in spite of the high RH (90-95%) used. Nevertheless no significant differences in weight loss were found between non-conditioned fruits and fruits conditioned for 1 or 2 days at 37 °C when they were subsequently exposed to the single or double quarantine treatments followed by the SL period. Our results also showed that these conditioning treatments did not significantly affect fruit firmness, colour or acidity after the SL period. The TSSC content and the maturity index slightly increased by 1 and 2 days at 37 °C but no relevant differences between non-conditioned and conditioned fruits were observed after SL in fruits exposed to the simple or the double quarantine treatments. These results are, in general, in concordance with those reported in blood orange cultivars conditioned for 2 days at 37 °C and stored for 16 days at 1.5 °C, though fruit weight loss and the occurrence of off-flavours, was increased by this heat treatment in blood oranges (Schirra, Mulas, Fadda, & Cauli, 2004). We did not detect off-flavours, which is in concordance with the lack of susceptibility of other mandarin fruit cultivars to develop off-flavour in response to high-temperature conditioning treatments (Pérez et al., 2005a; Pérez et al., 2005b).

3.2. Influence of high temperature-conditioning on flavonoids TAC and vitamin C of cold stored mandarins

Previous studies have shown that the application of very high temperatures (≥ 95 °C) may alter the concentration and composition of phenolic compounds of peel extracts and of processed juices derived from citrus fruit. (Xu, Ye, Chen, & Liu, 2007; Igual, García-Martínez, Camacho, & Martínez-Navarrete, 2010). However, knowledge on the effect of less extreme high temperatures (between 30 and 52 °C) used to increase the tolerance of citrus fruit to chilling on specific polyphenols is still essential to determine the impact of these treatments on their nutritional value.

To our knowledge, there is only one report evaluating changes in two specific flavonoids induced by a very short hot water dip treatment (2 min at 50 °C) in whole Kumquat fruits stored at a non-chilling temperature (17 °C) (Schirra et al., 2008). Moreover, the relationship between these changes and peel or pulp was not examined since in Kumquat the whole fruit is eaten. We have previously shown that the activity of the rate controlling enzyme in the synthesis of phenylpropanoids (phenylalanine ammonia-lyase, PAL) and the accumulation of *PAL* transcript may be altered in the peel of cold stored 'Fortune' mandarin by conditioning the fruit at 37 °C (Martínez-Téllez & Lafuente, 1997; Sánchez-Ballesta, Zacarías, Granell, & Lafuente, 2000), and that PAL activity increases in the peel of chilling sensitive citrus fruit cultivars that develop damage when stored at low temperature (Lafuente et al., 2003). Likewise, some flavonoids may increase in cold stored minimally processed segments but decrease in juices of different *Citrus* species (Del Caro, Piga, Vacca, & Aggabio, 2004). These findings suggest that stressful temperatures may alter phenylpropanoid metabolism in citrus fruits and that responses to temperature stress may differ among fruit tissues. Nevertheless, the impact of high and low temperatures in phenolics in the pulp of

257 intact whole fruits, which is protected by the peel and is not prone to oxidative processes
258 caused by loss of cellular compartmentalization like the juices, but still perceives temperature
259 stress, remains unknown. Therefore, and considering the above results on fruit quality, we
260 have evaluated the effect of conditioning 'Fortune' mandarins for just 1 day at 37 °C on the
261 concentration and composition of flavonoids, the most abundant phenolics in the pulp of
262 'Fortune' mandarins, and how they change during fruit cold storage. Eight flavonoids were
263 identified by comparison with the spectra and retention time of those of authentic standards.
264 The flavanone hesperidin was the most abundant flavonoid in the pulp of freshly harvested
265 fruit, followed by the flavone isorhoifolin and the flavanones didymin and narirutin (Fig. 2).
266 'Fortune' mandarins had also relative high levels of the flavanone eriocitrin and the flavone
267 rutin. Furthermore, the PMFs nobiletin and tangeretin were detected, although their
268 concentrations were very low as compared to the other flavonoids (Fig. 2). It is noteworthy
269 that these results are in concordance with the abundance of flavonoids reported in fruits of
270 'Dancy' tangerine and 'Clementine' mandarin (Nogata et al., 2006), as 'Fortune' mandarin is
271 a hybrid of these two mandarin cultivars. Only small differences in the concentration of
272 flavonoids have been found, in general, between freshly harvested fruits and conditioned
273 fruits either before cold storage or after the quarantine treatments and the SL period (Fig. 2).
274 Thus, differences in the concentration of flavonoids between heat conditioned and non-
275 conditioned fruits never exceeded a 1.4-fold increase or decrease by these storage periods. It
276 is noteworthy that conditioning the fruit at 37 °C favoured a significant transient increase in
277 eriocitrin and narirutin levels by 8 days of cold storage, which is in concordance with
278 previous findings of our group indicating that heating 'Fortune' mandarins at 37 °C may
279 transiently enhance the induction of different genes involved in phenylpropanoid metabolism
280 in chilled fruits (Sánchez-Ballesta et al., 2003). No relevant changes were found in the most

abundant flavonoids, hesperidin and isorhoifolin, and of rutin in response to cold both in conditioned and non-conditioned fruits. In contrast, a continuous increase in the concentration of eriocitrin, narirutin and didymin occurred at 1.5 °C in non-conditioned fruits. Such rise was more marked in the case of eriocitrin that showed a 4-fold increase during the double cold quarantine treatment (32 days), while narirutin and didymin only increased 1.5 times. Interestingly, hesperidin markedly increased in minimally processed citrus segments and decreased in juices kept at low temperature (Del Caro, Piga, Vacca, & Aggabio, 2004). Therefore, these authors suggested that the rise in hesperidin in cold stored segments might be related to damage caused during its preparation and/or the accumulation of ethylene, which induces PAL in citrus fruit, in the boxes containing the segments. The different behaviour found in the pulp of cold stored intact fruits in the present study may reinforce this idea. However, in view of our results, we cannot rule out the possibility that although PAL catalyze the first step on phenylpropanoid biosynthesis, other enzymes in the complex network of reactions responsible for the synthesis of different phenolics show different responses to environmental stressful temperatures. Moreover, and considering the differential cellular compartmentalization of phenolics and enzymes responsible for their oxidation (Pourcel et al., 2007), our results showing no relevant decrease in flavonoids in the pulp of cold stored fruits indicate that the decline in phenolics occurring in the juices at low temperature is more likely related to their oxidation because of loss of cellular compartmentalization caused by juice preparation than to temperature stress. The concentration of PMFs was very low in the pulp of citrus fruit as compared to other flavonoids (Fig. 2). Nevertheless, we paid attention to these compounds as they are almost exclusively found in citrus (Sendra, Navarro, & Izquierdo, 1988) and because they may be more active and act in the low micromolar range inhibiting several common diseases than hydroxylated compounds (Tripoli et al., 2007). The results

showed that PMFs decreased after the quarantine treatment, although significant differences were only found in nobiletin in conditioned fruits. Tangeretin showed a very slight increased in fruits held for 1 day at 37 °C but decreased after fruit transfer at 1.5 °C, reaching by 24 days similar levels to those found in non-conditioned fruits at this temperature.

Several analytical methods have been proposed for determining TAC of biological samples, though all of them present some limitations (Leong & Shui, 2002; Prior, Wu, & Schaich, 2005). The ABTS free radical decolouration and DPPH· free radical-scavenging assays are useful tools to evaluate antioxidant activities of different fruits (Gil et al., 2000; Leong & Shui, 2002), including orange, pomelos and lemon fruits. DPPH· is more sensitive to hydrophobic flavanones while the ABTS method is more sensitive to hydrophilic antioxidants like vitamin C (Del Caro, Piga, Vacca, & Aggabio, 2004). By using the radical DPPH· assay, we have shown that the antioxidant capacity was barely affected by the heat-conditioning treatment or the single and double quarantine treatments (Table 2). Therefore, global changes in flavonoids, the most abundant phenolics in ‘Fortune’ mandarin, together with the fact that the concentration of eriocitrin was very low as compared to other flavonoids that did not change in response to cold, would be in concordance with the effect of the heat treatment and cold storage on TAC measured by the DPPH· assay. In this context, it is interesting to note that HPLC examination of phenolics in the pulp of ‘Fortune’ mandarins confirmed that the concentrations of other class of phenolics such as coumarins or hydroxycinnamic acids were negligible as compared to that of major flavonoids and barely changed under the temperature regimes selected in the present study (data not shown). As vitamin C and phenolics are the major contributors to TAC in the pulp of blond citrus cultivars (Gardner, White, McPhail, & Duthie, 2000; Leong & Shui, 2002), we have also determined vitamin C and antioxidant capacity, measured by ABTS free radical assay, and

showed that they did not vary ($p \leq 0.05$) during fruit conditioning at 37 °C or in response to low temperature either in heat-conditioned or non-conditioned fruits (Table 2). This result is in concordance with those found in intact fruits of other citrus cultivars showing that quarantine and postharvest high temperature-conditioning treatments similar to those reported in the present work barely affected vitamin C (Schirra, Mulas, Fadda, & Cauli, 2004, Pérez et al., 2005). Similarly, storage at low temperature had no deleterious effect on vitamin C content of minimally processed citrus segments (Del Caro, Piga, Vacca, & Aggabio, 2004). This, together with the fact that vitamin C may decrease in juices stored for prolonged periods as the used in the present study (Igual, García-Martínez, Camacho, & Martínez-Navarrete, 2010), highlights the benefit of consuming fruits rather than their juices.

3.3. Relevance of flavonoids in carpellary membranes of mandarin segments

Carpellary membranes might have a special contribution to the human diet by consuming fresh citrus fruits. It is well known that the concentration and composition of flavonoids is tissue-dependent (Nogata et al., 2006) and that many physiological responses to external stimuli, including the synthesis of health-promoting compounds, may vary among tissues (Matsumoto et al., 2009). Therefore, we have examined the concentration of the identified flavonoids in the juice and in the carpellary membranes of the segments of cold-stored fruits. Our results showed that the concentration of most flavonoids was much higher in carpellary membrane than in the juice (Table 4). The concentration of the most abundant flavonoid hesperidin was similar in the isolated membranes and in the juice. However, the PMFs nobiletin and tangeretin, which are almost exclusively found in citrus, were mostly found in the carpellary membranes. A similar trend was found when we compared eriocitrin, narirutin and didymin levels, although differences were less marked (9-14 times). Therefore, because of the high differences in the concentration of the above mentioned flavonoids,

removal of carpellary membrane may account for important flavonoids losses in spite of the lower proportion of carpellary membranes (~ 11 % in 'Fortune' mandarins) with respect to the juice (~ 89 %). As shown in Table 4, consumption of fresh fruit (pulp) would double the intake of eriocitrin, narirutin, didymin, nobiletin and tangeretin as compared to that of same amount of juice and would barely affect the intake of isorhoifolin, hesperidin and rutin. From the identified flavonoids, only rutin and isorhoifolin were in higher concentration in the juice. While rutin was only detected in the juice, the concentration of isorhoifolin in the carpellary membranes was about 4-fold lower than in the juice. The same trend was observed in heat-conditioned fruit (data non-shown). These results are in concordance with the higher levels of flavonoids found in cold stored segments from orange fruits compared to their respective juices (Del Caro, Piga, Vacca, & Aggabio, 2004) and highlights the added benefit of consuming citrus fruit rather than their juices due to the loss of flavonoids when removing carpellary membranes during juice extraction.

4. Conclusions

Results of this study indicate that conditioning 'Fortune' mandarins fruit at 37 °C for 1 day reduces CI and has neither a deleterious effect on fruit quality nor on the composition and concentration of phenolics, vitamin C and TAC relevant for nutrition. Small differences in the concentration of flavonoids were found between non-conditioned and conditioned fruit after cold storage. The concentration of the most abundant flavonoids hesperidin and isorhoifolin did not increase after the single or double quarantine treatment, while that of other less abundant flavonoids (didymin, narirutin and eriocitrin) increased at 1.5 °C. Therefore, conditioning 'Fortune' mandarins at 37 °C for 1 day may be a useful tool to allow exportation of this highly sensitive chilling cultivar after application of quarantine treatments at very

restrictive low temperatures without losing nutritional value. In addition, our results showing the abundance of flavonoids in carpellary membrane highlights the added benefit of consuming fresh citrus fruit rather than their juices.

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References

- AOAC. (1990). In: Horwitz, E. (Ed.). *Official methods of analysis*. AOAC International.
- Alasalvar, C., Al-Farsi, M., Quantick, P. C., Shahidi, F., & Wiktorowicz, R. (2005). Effect of chill storage and modified atmosphere packaging (MAP) on antioxidant activity, anthocyanins, carotenoids, phenolics and sensory quality of ready-to-eat shredded orange and purple carrots. *Food Chemistry*, 89, 69-76.
- Ballester, A. R., Izquierdo, A., Lafuente, M. T., & González-Candelas, L. (2010). Biochemical and molecular characterization of induced resistance against *Penicillium digitatum* in citrus fruit. *Postharvest Biology and Technology*, 56, 31-38.
- Del Caro, A., Piga, A., Vacca, V., & Aggabio, M. (2004). Changes of flavonoids, vitamin C and antioxidant capacity in minimally processed citrus segments and juices during storage. *Food Chemistry*, 84, 99-105.
- Gardner, P. T., White, T. A. C., McPhail, D. B., & Duthie, G. G. (2000). The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. *Food Chemistry*, 68, 471-474.
- Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M., & Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry*, 48, 4581-4589.
- Girennavar, B., Jayaprakasha, G. K., & Patil, B. S. (2008). Influence of pre-and post-harvest factors and processing on the levels of furocoumarins in grapefruits (*Citrus paradisi* Macf.). *Food Chemistry*, 111, 387-392.
- Holland, N., Sala, J. M., Menezes, H. C., & Lafuente, M. T. (1999). Carbohydrate content and metabolism as related to maturity and chilling sensitivity of cv. Fortune mandarins. *Journal of Agricultural and Food Chemistry*, 47, 2513-2518.

415 Igual, M., García-Martínez, E., Camacho, M. M., & Martínez-Navarrete, N. (2010). Effect of
 416 thermal treatment and storage on the stability of organic acids and the functional value
 417 of grapefruit juice. *Food Chemistry*, 118, 291-299.

418 Lafuente, M. T., Zacarías, L., Martínez-Téllez, M.A., Sánchez-Ballesta, M.T., & Granell, A.
 419 (2003). Phenylalanine ammonia-lyase and ethylene in relation to chilling injury as
 420 affected by fruit age in citrus. *Postharvest Biology and Technology*, 29, 308-317.

421 Lafuente, M. T., & Zacarias, L. (2006). Postharvest physiological disorders in citrus fruit.
 422 *Stewart Postharvest Review*, 2, 1-9.

423 Leong, L. P. & Shui, G. (2002). An investigation of antioxidant capacity of fruits in
 424 Singapore markets. *Food Chemistry*, 76, 69-75.

425 Lo Scalzo, R., Iannoccari, T., Summa, C., & Rapisarda, P. (2004). Effect of thermal
 426 treatments on antioxidant and antiradical activity of blood orange juice. *Food*
 427 *Chemistry*, 85, 41-47.

428 Martínez-Téllez, M. A., & Lafuente, M. T. (1997). Effect of high temperature conditioning on
 429 ethylene, phenylalanine ammonia-lyase, peroxidase and polyphenol oxidase activities
 430 in flavedo of chilled Fortune mandarin fruit. *Journal of Plant Physiology*, 150, 674-678.

431 Matsumoto, H., Ikoma, Y., Kato, M., Nakajima, N., & Hasegawa, Y. (2009). Effect of
 432 postharvest temperature and ethylene on carotenoid accumulation in the flavedo and
 433 juice sacs of Satsuma mandarin (*Citrus unshiu* Marc.) fruit. *Journal of Agricultural and*
 434 *Food Chemistry*, 57, 4724-4732.

435 Mulas, M., & Schirra, M. (2007). The effect of heat conditioning treatments on the
 436 postharvest quality of horticultural crops. *Stewart Postharvest Review*, 3, 1-6.

437 Nogata, Y., Sakamoto, K., Shiratsuchi, H., Ishii, T., Yano, M., & Ohta, H. (2006). Flavonoid
 438 composition of fruit tissues of Citrus species. *Bioscience, Biotechnology, and*
 439 *Biochemistry*, 70, 178-192.

440 Pérez, A. G., Luaces, P., Oliva, J., Ríos, J. J., & Sanz, C. (2005a). Changes in vitamin C and
 441 flavor component of mandarin juice due to curing of fruits. *Food Chemistry*, 91, 19-24.

442 Pérez, A. G., Luaces, P., Olmo, M., Sanz, C., & García, J. M. (2005b). Effect of intermittent
 443 curing on mandarin quality. *Journal of Food Science*, 70, 64-68.

444 Pourcel, L., Routaboul, J. M., Cheynier, V., Lepiniec, L., & Debeaujon I. (2007). Flavonoid
 445 oxidation in plants: from biochemical properties to physiological functions. *Trends in*
 446 *Plant Science*, 12, 29-36.

447 Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of
 448 antioxidant capacity and phenolics in foods and dietary supplements. *Journal of*
 449 *Agricultural and Food Chemistry*, 53, 4290-4302.

450 Rapisarda, P., Bellomo, S. E., Fabroni, S., & Russo, G. (2008). Juice quality of two new
 451 mandarin-like hybrids (*Citrus clementina* Hort. ex Tan x *Citrus sinensis* L. Osbeck)
 452 containing anthocyanins. *Journal of Agricultural and Food Chemistry*, 56(6), 2074-
 453 2078.

454 Rice-Evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M., & Pridham, J. B. (1995). The
 455 relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical*
 456 *Research*, 22, 375-383 .

457 Sánchez-Ballesta, M. T., Lluch, Y., Gosalbes, M. J., Zacarías, L., Granell, A., & Lafuente, M.
 458 T. (2003). A survey of genes differentially expressed during long-term heat-induced
 459 chilling tolerance in citrus fruit. *Planta*, 218, 65-70.

460 Sánchez-Ballesta, M. T., Zacarías, L., Granell, A., & Lafuente, M. T. (2000). Accumulation
 461 of Pal transcript and Pal activity as affected by heat-conditioning and low-temperature
 462 storage and its relation to chilling sensitivity in Mandarin fruits. *Journal of Agricultural*
 463 *and Food Chemistry*, 48, 2726-2731.

464 Schirra, M., Mulas, M., Fadda, A., & Cauli, E. (2004). Cold quarantine responses of blood
 465 oranges to postharvest hot water and hot air treatments. *Postharvest Biology and*
 466 *Technology*, 31, 191-200.

467 Schirra, M., Palma, A., D'Aquino, S., Angioni, A., Minello, E. V., Melis, M., & Cabras, P.
 468 (2008). Influence of postharvest hot water treatment on nutritional and functional
 469 properties of Kumquat (*Fortunella japonica* Lour. Swingle cv. Ovale) fruit. *Journal of*
 470 *Agricultural and Food Chemistry*, 56, 455-460.

471 Sendra, J. M., Navarro, J. L., & Izquierdo, L. (1988). C18 solid-phase isolation and high-
 472 performance liquid chromatography/ultraviolet diode array determination of fully
 473 methoxylated flavones in citrus juices. *Journal of Chromatographic Science*, 26, 443-
 474 448.

475 Sentandreu, E., Navarro, J. L., & Sendra, J. M. (2007). Effect of technological processes and
 476 storage on flavonoids content and total, cumulative fast-kinetics and cumulative slow-
 477 kinetics antiradical activities of citrus juices. *European Food Research and Technology*,
 478 225, 905-912.

479 Tripoli, E., La Guardia, M., Giammanco, S., Di Majo, D., & Giammanco, M. (2007). Citrus
 480 *flavonoids*: Molecular structure, biological activity and nutritional properties: A review.
 481 *Food Chemistry*, 104, 466-479.

482 Vanamala, J., Cobb, G., Loaiza, J., Yoo, K., Pike, L. M., Patil, B. S. (2007). Ionizing radiation
483 and marketing simulation on bioactive compounds and quality of grapefruit (Citrus
484 paradisi c.v. Rio Red). *Food Chemistry*, 105, 1404-1411.

485 Xu, G., Ye, X., Chen, J., & Liu, D. (2007). Effect of heat treatment on the phenolic
486 compounds and antioxidant capacity of citrus peel extracts. *Journal of Agricultural and*
487 *Food Chemistry*, 55, 330-335.

488

Figure captions

Fig. 1. Changes in CI index of ‘Fortune’ mandarins stored for 16 and 32 days at 1.5 °C and a subsequent SL period of 4 days at 20 °C. Freshly harvested fruit were stored immediately at 1.5 °C (black bar) or after being conditioned for 1 (grey bar) or 2 (white bar) days at 37 °C. Results represent the means of three replicates samples of ten fruits each. Values labeled with the same letter within the same storage conditions (group of bars) are not different at the 5% significance level according to Tukey’s test.

Fig. 2. Concentrations ($\mu\text{g g}^{-1}$ FW) of the main flavonoids identified in the pulp of ‘Fortune’ mandarins fruits. Freshly harvested fruit were stored immediately at 1.5 °C (●) or after being conditioned for 1 day at 37 °C (○) and then transferred for 4 days to air at 20 °C. Dotted lines and arrows in the plots indicate the transfer to 20 °C. Hesperidin (HSP), isorhoifolin (IRF), didymin (DID), narirutin (NRT), eriocitrin (ERC), rutin (RTN), nobiletin (NBT), and tangeretin (TGT). Note the different scale for each flavonoid. Results represent the means of three replicates samples of ten fruits each $\pm$ SEM.

Table 1

Effect of conditioning ‘Fortune’ mandarins for 1 or 2 days at 37 °C and 90-95% RH on changes in fruit quality during cold storage and a subsequent SL period of 4 days at 20 °C

	Control fruit (non-conditioned)	Conditioning 1d 37 °C	Conditioning 2d at 37 °C
Weight loss (%)			
0d 1.5 °C	0.00 ^b	1.65 ^a	2.64 ^a
16d 1.5 °C	2.58 ^b	4.45 ^a	3.94 ^a
16d 1.5 °C + 4d 20 °C	8.06 ^a	7.38 ^a	8.14 ^a
32d 1.5 °C	5.34 ^b	6.05 ^a	6.43 ^a
32d 1.5 °C + 4d 20 °C	10.51 ^a	9.71 ^a	9.97 ^a
Fruit firmness (Kg)			
0d 1.5 °C	4.22 ^b	5.77 ^a	4.80 ^{ab}
16d 1.5 °C	4.29 ^b	5.84 ^a	3.91 ^b
16d 1.5 °C + 4d 20 °C	5.23 ^a	4.97 ^{ab}	5.47 ^a
32d 1.5 °C	6.51 ^a	5.55 ^b	5.84 ^{ab}
32d 1.5 °C + 4d 20 °C	5.91 ^a	6.11 ^a	5.69 ^a
Fruit colour (IC)			
0d 1.5 °C	16.83 ^a	19.05 ^a	16.79 ^a
16d 1.5 °C	19.49 ^a	19.70 ^a	17.73 ^a
16d 1.5 °C + 4d 20 °C	20.79 ^a	19.09 ^a	19.70 ^a
32d 1.5 °C	17.68 ^a	18.98 ^a	17.70 ^a
32d 1.5 °C + 4d 20 °C	19.99 ^a	19.10 ^a	17.16 ^a
Acidity (% citric acid)			
0d 1.5 °C	1.72 ^a	1.66 ^a	1.65 ^a
16d 1.5 °C	1.87 ^a	1.55 ^{ab}	1.45 ^b
16d 1.5 °C + 4d 20 °C	1.75 ^a	1.61 ^b	1.72 ^{ab}
32d 1.5 °C	1.63 ^a	1.54 ^a	1.64 ^a
32d 1.5 °C + 4d 20 °C	1.37 ^b	1.66 ^a	1.44 ^{ab}
TSSC (°Brix)			
0d 1.5 °C	13.40 ^b	14.23 ^a	14.57 ^a
16d 1.5 °C	14.16 ^{ab}	13.46 ^b	14.71 ^a
16d 1.5 °C + 4d 20 °C	15.13 ^a	14.50 ^b	15.23 ^a
32d 1.5 °C	14.63 ^a	14.74 ^a	15.18 ^a
32d 1.5 °C + 4d 20 °C	14.12 ^a	14.46 ^a	14.70 ^a
Maturity index			
0d 1.5 °C	7.79 ^b	8.57 ^a	8.83 ^a
16d 1.5 °C	7.57 ^c	8.68 ^b	10.14 ^a
16d 1.5 °C + 4d 20 °C	8.65 ^a	9.00 ^a	8.85 ^a
32d 1.5 °C	8.98 ^b	9.57 ^a	9.26 ^{ab}
32d 1.5 °C + 4d 20 °C	10.31 ^a	8.71 ^b	10.21 ^a

Results represent the means of three replicates samples. Values labeled with the same letter within the same row are not different at the 5% significance level according to Tukey’s test.

Table 2. Effect of conditioning ‘Fortune’ mandarins for 1 day at 37°C and 90-95% RH on changes in TAC, measured by the DPPH· (%) and the ABTS (meq Trolox mL⁻¹) assays, and in vitamin C in the pulp of ‘Fortune’ mandarins stored for 16 and 32 days at 1.5 °C and 90-95% RH and a subsequent SL period of 4 days at 20°C.

	Control fruit (non-conditioned)	Conditioning 1d 37°C
DPPH· (%)		
0d 1.5 °C	22.71 ^a	19.57 ^a
16d 1.5 °C	20.20 ^a	19.05 ^a
32d 1.5 °C	19.41 ^a	21.43 ^a
32d 1.5°C + 4d 20 °C	21.30 ^a	21.70 ^a
TAC (meq Trolox mL ⁻¹)		
0d 1.5 °C	1.72 ^a	1.59 ^a
16d 1.5 °C	1.62 ^a	1.60 ^a
32d 1.5 °C	1.58 ^a	1.57 ^a
32d 1.5°C + 4d 20 °C	1.52 ^a	1.51 ^a
Vitamin C (mg 100 mL ⁻¹)		
0d 1.5 °C	30.40 ^a	30.80 ^a
16d 1.5 °C	29.59 ^a	28.25 ^a
32d 1.5 °C	31.55 ^a	28.92 ^a
32d 1.5°C + 4d 20 °C	31.32 ^a	29.62 ^a

Results represent the means of three replicates samples. Values labeled with the same letter are not different at the 5% significance level according to Tukey’s test

Table 3

Flavonoids contents in carpellary membranes, juice and pulp of 'Fortune' mandarins stored for 32 days at 1.5 °C and a subsequent SL period of 4 days at 20 °C. Hesperidin (HSP), isorhoifolin (IRF), didymin (DID), narirutin (NRT), eriocitrin (ERC), rutin (RTN), nobiletin (NBT) and tangeretin (TGT). Results represent the means of three replicates samples $\pm$ SEM

Flavonoid	Membrane $\mu\text{g g}^{-1}\text{FW}$	Juice $\mu\text{g g}^{-1}\text{FW}$	Pulp $\mu\text{g g}^{-1}\text{FW}$	Fold pulp/juice
ERC	40.82 $\pm$ 8.35	3.01 $\pm$ 0.04	7.17 $\pm$ 0.93	2.38
RTN	nd	16.09 $\pm$ 0.18	15.91 $\pm$ 0.30	0.99
NRT	1601 $\pm$ 19	159 $\pm$ 1	275 $\pm$ 29	1.79
IRF	112 $\pm$ 12	453 $\pm$ 5	482 $\pm$ 29	1.06
HSP	927 $\pm$ 22	1230 $\pm$ 59	1469 $\pm$ 172	0.97
DID	2155 $\pm$ 34	240 $\pm$ 11	485 $\pm$ 60	1.19
NBT	1.044 $\pm$ 0.097	0.078 $\pm$ 0.002	0.184 $\pm$ 0.070	2.37
TGT	0.816 $\pm$ 0.049	0.050 $\pm$ 0.008	0.097 $\pm$ 0.046	1.93

nd: non detected.

Figure 1

Figure 2 revised FOODCHEM