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To cite this article: Angelo Maria Giuffrè, Clotilde Zappia & Marco Capocasale (2017) Physicochemical stability of blood orange juice during frozen storage, International Journal of Food Properties, 20:sup2, 1930-1943

To link to this article: https://doi.org/10.1080/10942912.2017.1359184
Physicochemical stability of blood orange juice during frozen storage

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ABSTRACT
Blood orange juice from Citrus sinensis (L.) Osbeck cv. Moro was compared to a concentrated blood orange juice and both juices were studied during frozen storage at −20°C. Analyses were conducted on pH (3.33–3.81) and titratable acidity as citric acid (11.14–13.16 g/L). The formol number decreased in both blood orange juice and concentrated blood orange juice during frozen storage, while vitamin C showed a very slight decrease. Scavenging abilities of the juices for the DPPH ∙ radical ranged from 53.39% to 42.55% in blood orange juice and from 39.16% to 33.75% in concentrated blood orange juice during frozen storage. Although anthocyanins showed a diminution during storage in both the concentrated and non-concentrated blood orange juice, they were always higher in the non-concentrated juice fruit. Four phenolic acids were detected: gallic, chlorogenic (the highest quantity, 13–27 mg/L), caffeic, and ferulic, the latter showed the lowest content. Ten flavonoids were identified, two flavonols (rutin and quercetin) and eight flavanones: narirutin (the second highest flavonoid), naringin, hesperidin (the highest quantity), neoeriocitrin, didymin, eriocitrin, neohesperidin, and hesperetin. Concentration and duration of frozen storage were found to influence the physicochemical properties of blood orange juice in different ways.

ARTICLE HISTORY
Received 19 January 2017
Accepted 20 July 2017

KEYWORDS
Anthocyanins; Citrus; European Fruit Juice Association; Flavonoids; Freezing; Nutraceuticals; Orange juice; Phenolic acids

Introduction
South Italy has a tradition of citrus cultivation. In 2013–2014, Italy produced 1,935,000 tonnes of orange fruit, whereas the major producers were Brazil (16,850,000 tonnes) and China (7,600,000 tonnes).\(^1\) Citrus juice is normally appreciated by consumers for its beneficial effects on human health and its vitamin C content.\(^2\) Orange juice from Citrus sinensis (L.) is rich in vitamin C (ascorbic acid) and in other antioxidant compounds.\(^3\) It is one of the most widely recognized functional foods and has been widely studied. Citrus juice induces changes in the human metabalome linked to the steroid biosynthesis pathway; therefore, a dietary dose of citrus juice was able to produce short-term effects on endogenous metabolites without requiring its chronic intake.\(^4\) Drinking orange juice (500 mL/day) increases plasma concentrations of vitamin C and reduces concentrations of 8-epi-prostaglandin F (2 alpha) (8-epi-PGF (2 alpha)) in humans; these effects were significantly more pronounced in smokers.\(^5\) The administration of citrus juice has shown beneficial effects on certain parameters of the lipid profile, with a beneficial reduction in plasmatic cholesterol, High-Density Lipoproteins - cholesterol (HDL-c), and Low Density Lipoproteins - cholesterol (LDL-c) in metabolic syndrome compared to the baseline values. Also, improved values of ox-LDL, C-reactive protein, and homocysteine levels have been found after 6 months of consumption.\(^2\)

The need to sell orange juice on the world market, the continuous evolution of the food industry, the evolution of consumer choice, which requires food products to appear as natural as possible, all determine the necessity to study minimally processed systems to store the juice and maintain its physicochemical properties.\(^6\) For producers, it is very important to extend all positive properties of
blood orange juice (BOJ) (as far as possible) during storage. In order to prevent misleading advertising, the manufacturer should evaluate the shelf life of the product depending on the ingredients used, their concentration, and their durability.\[7\] Studies have been conducted to improve or extend citrus juice shelf-life by pulsed electric field\[8\], by ultrafiltration\[9\], by reverse osmosis and osmotic distillation.\[10\] Beltrán et al.\[11\] studied the vitamin C content in mandarin orange (Citrus reticulata) juice packed in two different non-transparent cartons made of polyethylene and at different temperatures, and found a common decreasing trend in all samples but with a different rate according to the duration of storage, packaging, and temperature. The aim of this work was to study the influence of freezing and frozen storage on the physicochemical properties of BOJ and concentrated blood orange juice (CBOJ). The orange juice was frozen to maintain as long as possible its properties, and it was diluted to 11.2 °Brix to be analysed as indicated by the European Fruit Juice Association (AIJN).\[12\]

**Materials and methods**

**Fruit and juice material and analytical criterion**

C. sinensis (L.) cv. Moro, a blood orange widely grown in Sicily on Citrus aurantium (bitter orange) as a rootstock, was used in this experiment. The commercial orange tree orchard (20 ha) was located near Catania, Sicily, South Italy. The climate, dry with infrequent rain, is characterized by hot summers and mild winters with a temperature variation of 12°C between day and night in autumn–winter. The variation in temperature during fruit ripening (autumn–winter) increases the aptitude of the BOJ cultivars to produce anthocyanins: the larger the variation, the higher the anthocyanin content. In more detail, the north side of the fruit became intensely red if compared with the scarcely red or orange colour of its south side.

The same plants cultivated in a different geographical area do not accumulate the same anthocyanin content. The trees (20 years old in March 2015) had been planted in rows 5 m apart, with a distance of 5 m between trees. They were irrigated on the basis of their evapotranspiration. Commercial maturity of fruits was assessed at 10.50 ± 0.80 °Brix (this value was calculated on 50 fruits). Healthy fruits were manually picked on 15 March 2015; they were carefully placed in plastic containers (20 kg per container) and stored in a warehouse at 12°C for 2–3 h. At this point, the fruits were transferred to the factory for juice extraction. BOJ was obtained by the following procedure: (i) orange collection; (i), orange washing in a tank with water; (iii) orange transportation by a conveyor-belt; (iv) cutting in two halves; (v) squeezing; (vi) juice–pulp centrifugation; (vii) juice pasteurization; and (viii) juice concentration. A SPECIALE equipment model SP40 (Catania, Italy), for citrus juice extraction was used. After squeezing, the obtained juice–pulp mixture was introduced into a horizontal decanter to reduce the pulp content to 2–3% of the total extract. The orange juice was pasteurized at 92°C for 32 s by a plate pasteurizer. Following this, a SANTORO five-effect falling film evaporator equipment (Catania, Italy) was used to concentrate the juice from 10.50 to 55 °Brix. The concentration temperature was managed under vacuum from 95°C in the first effect to 30°C in the fifth effect, and the pressure changed from 0.74 to 0.30 atm from the first to the fifth effect.

Two types of BOJ were prepared for the experiment: a BOJ and a concentrated (55 °Brix) BOJ. In the laboratory, both BOJ and CBOJ were diluted to 11.2 °Brix as required by the AIJN\[12\] and immediately analysed (T0). The AIJN\[12\] lists the acceptability parameters for orange juice when the juice is at 11.2 °Brix. Other aliquots of BOJ and CBOJ (11.2 °Brix) were frozen at −20°C for 5 months in 100 mL polyethylene flasks and analysed monthly (T1, T2, T3, T4, and T5). Three samples of each juice were analysed at each month of storage. In this work, the analyses performed were chosen on the basis of the relevant European regulations, as well as the parameters required by the orange juice industry.
Chemicals

All reagents (analytical and HPLC grade) were purchased from VWR International (Milan, Italy). Analytical standards (purity >97%) were from Extrasynthese (Lyon, France).

Physicochemical properties

This potentiometric analysis was conducted by a pH meter model Crison basic 20 (Barcelona, Spain). Brix was determined by a digital refractometer Atago PR-101α (Tokyo, Japan). The titratable acidity (TA) was determined by the IFUMA03 method. Two grams of juice and 50 mL of deionized water were placed into a glass beaker and the solution was titrated to a pH of 8.1 with a 0.25 N NaOH solution. The result was expressed as g/L of citric acid monohydrate.

Vitamin C

Vitamin C quantification was carried out using an iodometric titration. Also, 10 mL of juice and 5 mL of deionized water were placed into a beaker. The solution was titrated with a 0.01 N iodine solution using a 2% starch solution as an indicator. The result is expressed as mg ascorbic acid/L juice. The colour of the orange juice, even if diluted with water, interferes with the iodine-starch system, making it difficult to detect the exact point of toning. For this reason, the mixture was exposed to an intense light during titration to help distinguish the red colour of the juice from the purple colour of the titrated solution. An additional aliquot of orange juice was placed in a beaker next to the juice to be analysed, to make the point of toning more evident.

Formol number

The formol number was determined with the method IFUMA30. Also, 10 mL of juice and 10 mL of 40% by volume formaldehyde solution (pH 2.8) were placed in a beaker and phenolphthalein as the indicator was added. The mixture was titrated with a 0.1 NaOH solution until pH 8.2. The formol number was calculated with the following formula: mL NaOH used for titration/2.

Antioxidant activity

DPPH· methodology is based on the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH·), which strongly absorbs at 515 nm on the corresponding hydrazine by the free radical scavenging activity of the antiradical. The antiradical activity of orange juice was carried out using a solution of $6 \times 10^{-5}$ M DPPH· in methanol and reading using a spectrophotometer Agilent spectrophotometer (CA, USA) model 89090A at 515 nm. A 2.5 mL aliquot of $6 \times 10^{-5}$ M DPPH· in methanol was placed in a quartz cuvette and the absorbance was read at $t_0$ min. At this point 10 μL of juice was placed in the cuvette maintaining a constant agitation for 5 min and the absorbance was read again ($t_5$ min). The result is expressed as % reduction of DPPH· by the following formula:

$$ \% \text{ DPPH·} = 100 \times (1 - (t_5 / t_0)). $$

Total phenols

The orange juice was centrifuged at 10,000 rpm for 5 min, and the supernatant was used to determine the total phenolic content and the antioxidant activity by DPPH· assay. The analysis of total phenols was performed following the colorimetric method of Folin–Ciocalteu. Into a 25 mL glass flask were placed 0.2 mL juice, 5 mL deionized water, and 1 mL Folin–Ciocalteu reagent. After 8 min, 10 mL of 20% Na$_2$CO$_3$ solution was added and the mixture was made up to volume with
deionized water. At the same time, a reference solution (blank) was prepared using the same amount of reagents but without the sample. The mixtures were left in the dark for 2 h before reading the absorbance at 765nm using an Agilent spectrophotometer (CA, USA) model 89090A. The total phenol content was expressed as mg of gallic acid/L of juice.

**Total anthocyanic content**

Anthocyanins were analysed using the pH differential method no. 2 suggested by Rapisarda et al.\textsuperscript{[18]} Results were expressed as mg/L of cyaniding 3-glucoside chloride. Spectrophotometric analysis was conducted using a Perkin Elmer instrument (Waltham, MA, USA) model Lambda 2.

**Phenolic acids and flavonoids**

The orange juice was centrifuged at 10,000 rpm (5 min) and filtered through a 0.45 μm particle size and 25 mm diameter filter (Chromafil RC-45/25, Macherey-Nagel GmbH & Co. KG) before injection (20 μL) directly into the HPLC.\textsuperscript{[17]-[19]} The HPLC analysis was carried out using a Knauer instrument (Berlin, Germany) equipped with a DAD detector (model 2600); the selected wavelengths were 280 nm for gallic acid, eriocitrin, neo-eriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin, and hesperetin (Figure 1); 305 nm for chlorogenic acid, caffeic acid, and ferulic acid (Figure 2) and 365 nm for quercetin and rutin (Figure 3). HPLC-DAD is commonly used in the study of antioxidants in other matrices, such as grape berry skin\textsuperscript{[20]}, sweet orange peels\textsuperscript{[21]}, and virgin olive oil.\textsuperscript{[22]}

Elution was with a binary gradient: bi-deionized water (mobile phase A) and acetonitrile (mobile phase B), both mobile phases were acidified (pH 3) with formic acid. Analyses were performed using the following gradient: 95% A and 5% B in isocratic (20 min); eluent B increased from 5% to 40% from 20 to 50 min; from 40% to 95% from 50 to 60 min; and then decreased from 95% to 5% from 60 to 65 min and finally 95% A and 5% B in isocratic from 65 to 70 min at 1 mL/min flow rate. The separation column was a Knauer C18 Eurosphere II with pre-column (250 mm length χ 4.6 mm internal diameter χ 5 μm particle size). Phenolic compounds were quantified with the external standard technique and using a standard solution containing all the detected phenols.

![Figure 1. HPLC-DAD chromatogram of gallic acid and flavanons in the blood orange juice at 280 nm.](image-url)
HPLC method validation

Selectivity
Each standard compound was singly injected and the retention time was noted. Afterwards, a standard solution combining all the different standards was injected and the retention times were compared between each single injection and the combined standards solution injection. No co-eluting peaks were detected.

Linearity
The combined standard solutions were prepared at five different concentrations and injected into the chromatographic system. The correlation coefficient was calculated in the range of 0.9970–0.9981 for phenolic acids and in the range of 0.9984–0.9989 for flavonoids.
Limits of detection and quantification

The limit of detection varied from 0.01 to 0.03 mg/L for phenolic acids and between 0.01 and 0.02 mg/L for flavonoids. The limit of quantification varied from 0.03 to 0.05 mg/L for both phenolic acids and flavonoids.

Statistical analysis

Analyses were conducted in triplicate; means and standard deviations were calculated by the Excel software (2007 version). One-way ANOVA was applied to the different storing date using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA); differences between sample means were analysed by Tukey’s method of multiple comparison at \( p < 0.05 \).

Results and discussion

\( pH \)

\( pH \) showed a slight tendency to decrease for both BOJ and CBOJ during frozen storage (Table 2). The highest values were found in \( T_0 \) (3.74 and 3.81, respectively, for BOJ and CBOJ). After 5 months’ storage, \( pH \) values decreased to 3.34 (BOJ) and 3.50 (CBOJ) (Table 2). Similar data were found in cv. Navel (3.7), in cv. Tarocco (3.6), in cv. Moro (3.6), and in cv. Sanguinello (3.6) from Sicily.\(^{[23]} \) Other authors found similar values with a slight increase from 3.34 to 3.43 in the \( pH \) value in non-processed orange juice during 12 days storage at 4°C.\(^{[24]} \)

\( Brix \)

This measure represents the level of total soluble solids in a given sample, the system previously having been calibrated with sucrose solutions at known concentrations. \( °Brix \) was similar in both BOJ and CBOJ during 5 months of storage and ranged from 10.7 to 11.5 (Table 2). In all cases, Brix values were above 10.0 (Table 1): the minimum indicated by the AIJN.\(^{[12]} \) Destani et al.\(^{[25]} \) found 11.0 °Brix in squeezed, depectinized, and filtered BOJ cv. Tarocco from Calabria (South Italy). In squeezed and centrifuged orange juices, Ingallinera et al.\(^{[23]} \) found a 13.4 °Brix in cv. Navel, 13.5 °Brix in cv. Tarocco, 11.6 °Brix in cv. Moro, and 12.6 °Brix in cv. Sanguinello from Sicily.\(^{[23]} \) Other authors found lower °Brix values in non-sonicated (8.9 °Brix) and sonicated (8.8–8.9 °Brix) fresh filtered orange juice of \( C. \) sinensis cv. Valencia from Spain.\(^{[26]} \)

\( Titratable acidity \)

Acidity increased in the BOJ over the 5 months’ storage, from 12.66 in \( T_0 \) to 13.16 g/L in \( T_5 \) (0.5 acidity more, i.e. 3.94% increase). Similar results were found in CBOJ, from 11.16 g/L in \( T_0 \) to 11.60 g/L in \( T_5 \) (0.44 g/L more acidity, i.e. 3.94% increase) (Table 2). All values were in the range indicated by the AIJN,\(^{[12]} \) 5.8–15.4 g/L (Table 1). The TA evolution found in the studied samples confirmed the \( pH \) decrease, the higher the acidity, the lower the \( pH \) value. Tiwari et al.\(^{[26]} \), in squeezed, filtered, and sonicated juice of \( C. \) sinensis cv. Valencia from Spain, found 6.3 g/L as a TA, half of that in our samples. Kelebek et al.\(^{[27]} \), in squeezed and centrifuged BOJ, found 11.3 and 13.4 g/L as TA after freezing at −18°C (cv. Moro and Sanguinello, respectively). Palma et al.\(^{[28]} \) found

<table>
<thead>
<tr>
<th>Brix (BOJ)</th>
<th>Brix (CBOJ)</th>
<th>Titratable acidity (g/L citric acid)</th>
<th>Vitamin C (mg/L)</th>
<th>Formol number (mL NaOH 0.1 N /100 mL)</th>
<th>Hesperidin (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min.</td>
<td>Min.</td>
<td>Range</td>
<td>Min.</td>
<td>Range</td>
<td>Range</td>
</tr>
<tr>
<td>10.0</td>
<td>11.2</td>
<td>5.8–15.4</td>
<td>200</td>
<td>15–26</td>
<td>250–700</td>
</tr>
</tbody>
</table>
Table 2. Physicochemical parameters of BOJ and CBOJ.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>°Brix</th>
<th>Acidity (g/L citric acid)</th>
<th>Vitamin C (mg/L)</th>
<th>Formol number (mL NaOH 0.1 N /100 mL)</th>
<th>AA DPPH-assay (%)</th>
<th>Total phenols (mg/L)</th>
<th>Total anthocyanins (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_0$</td>
<td>3.74 ± 0.03 b</td>
<td>11.1 ± 0.1 b</td>
<td>12.7 ± 0.4 a</td>
<td>679.8 ± 15.4 a</td>
<td>17.3 ± 0.3 a</td>
<td>53.4 ± 1.5 a</td>
<td>249.5 ± 11.1 abcde</td>
<td>143.6 ± 0.5 a</td>
</tr>
<tr>
<td>$T_1$</td>
<td>3.65 ± 0.05 b</td>
<td>11.1 ± 0.0 b</td>
<td>12.2 ± 0.8 ab</td>
<td>594.3 ± 8.8 e</td>
<td>16.2 ± 0.1 b</td>
<td>51.0 ± 2.7 a</td>
<td>249.4 ± 8.3 abcd</td>
<td>143.1 ± 0.5 a</td>
</tr>
<tr>
<td>$T_2$</td>
<td>3.33 ± 0.03 d</td>
<td>10.7 ± 0.1 d</td>
<td>12.5 ± 0.2 ab</td>
<td>600.6 ± 2.2 de</td>
<td>15.4 ± 0.1 d</td>
<td>43.6 ± 2.2 bc</td>
<td>250.4 ± 5.2 abcde</td>
<td>143.0 ± 0.2 a</td>
</tr>
<tr>
<td>$T_3$</td>
<td>3.72 ± 0.07 ab</td>
<td>10.9 ± 0.1 d</td>
<td>12.9 ± 0.2 a</td>
<td>602.8 ± 4.4 de</td>
<td>16.5 ± 0.3 cd</td>
<td>44.8 ± 0.1 b</td>
<td>241.5 ± 7.4 cde</td>
<td>143.1 ± 0.55 a</td>
</tr>
<tr>
<td>$T_4$</td>
<td>3.34 ± 0.01 d</td>
<td>10.8 ± 0.7 cd</td>
<td>12.8 ± 0.1 a</td>
<td>644.4 ± 2.2 b</td>
<td>15.2 ± 0.2 d</td>
<td>43.0 ± 0.7 bc</td>
<td>230.9 ± 2.1 e</td>
<td>142.9 ± 0.1 a</td>
</tr>
<tr>
<td>$T_5$</td>
<td>3.34 ± 0.04 d</td>
<td>10.8 ± 0.1 cd</td>
<td>13.2 ± 0.4 a</td>
<td>605.3 ± 6.5 g</td>
<td>15.2 ± 0.1 d</td>
<td>42.6 ± 0.4 bc</td>
<td>265.7 ± 9.3 ab</td>
<td>142.9 ± 0.1 a</td>
</tr>
<tr>
<td>CBOJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_0$</td>
<td>3.81 ± 0.03 a</td>
<td>11.2 ± 0.1 b</td>
<td>11.2 ± 0.2 c</td>
<td>621.3 ± 15.4 bcd</td>
<td>16.0 ± 0.3 bc</td>
<td>39.2 ± 2.7 cd</td>
<td>247.5 ± 7.0 bcde</td>
<td>129.7 ± 0.7 b</td>
</tr>
<tr>
<td>$T_1$</td>
<td>3.73 ± 0.03 ab</td>
<td>11.5 ± 0.1 a</td>
<td>11.2 ± 0.2 c</td>
<td>627.0 ± 2.2 bc</td>
<td>15.3 ± 0.4 d</td>
<td>36.7 ± 2.9 d</td>
<td>267.7 ± 2.1 a</td>
<td>129.0 ± 0.6 bc</td>
</tr>
<tr>
<td>$T_2$</td>
<td>3.43 ± 0.03 cd</td>
<td>11.1 ± 0.1 b</td>
<td>11.6 ± 0.2 bc</td>
<td>589.7 ± 4.4 ef</td>
<td>15.2 ± 0.1 d</td>
<td>35.2 ± 3.0 d</td>
<td>266.7 ± 4.2 ab</td>
<td>127.6 ± 0.6 c</td>
</tr>
<tr>
<td>$T_3$</td>
<td>3.44 ± 0.05 cd</td>
<td>10.8 ± 0.1 cd</td>
<td>11.1 ± 0.2 c</td>
<td>567.3 ± 0.6 f</td>
<td>15.2 ± 0.3 d</td>
<td>35.7 ± 1.3 d</td>
<td>235.3 ± 7.4 de</td>
<td>124.8 ± 0.6 d</td>
</tr>
<tr>
<td>$T_4$</td>
<td>3.47 ± 0.07 c</td>
<td>11.0 ± 0.1 bc</td>
<td>11.6 ± 0.2 bc</td>
<td>607.1 ± 4.4 cde</td>
<td>15.1 ± 0.1 d</td>
<td>34.8 ± 0.7 d</td>
<td>252.2 ± 5.9 abcd</td>
<td>122.7 ± 1.3 e</td>
</tr>
<tr>
<td>$T_5$</td>
<td>3.50 ± 0.01 c</td>
<td>11.0 ± 0.2 bc</td>
<td>11.6 ± 0.2 bc</td>
<td>591.4 ± 5.8 h</td>
<td>15.1 ± 0.1 d</td>
<td>33.8 ± 0.8 d</td>
<td>258.4 ± 2.7 abc</td>
<td>122.2 ± 0.3 e</td>
</tr>
</tbody>
</table>

Significance *** *** *** *** *** **

Different letters in the same column indicate statistically significant differences.

*"p < 0.01; ***"p < 0.001.
13.8 g/L in cv. Tarocco juice from South Sardinia (Italy); they also found a slight decrease in TA content after hot water postharvest treatments (from 20 to 59°C) and after simulated quarantine conditions for fruit disinfection. In cv. Moro juice, Lo Scalzo et al.\(^{29}\) found an effect in TA content caused by thermal treatments; they found 106 meq/100 g in untreated orange juice; 80.1 meq/100 g in squeezed segments previously blanched for 6 min at 80°C; 101 meq/100 g in squeezed segments pasteurized at 80°C for 1 min.

**Vitamin C**

Vitamin C is found almost exclusively in foods of plant origin. Its absorption in the human body occurs in the buccal mucosa, stomach, and small intestine. Buccal absorption is thought to be by way of passive diffusion through the membrane of the buccal mucosal cells. Gastrointestinal absorption of the vitamin is rapid and efficient, and an active carrier-mediated transport system has been suggested, especially at low concentrations.\(^{30}\) In oranges, the predominant form of vitamin C is ascorbic acid, whereas dehydroascorbic acid is less than 10% of total vitamin C.\(^{3}\) It is a water-soluble molecule that cannot be produced by humans but has to be ingested in the diet.\(^{31}\) The National Institute of Health recommends a daily intake of 15 mg/day for 1–3-year-old children up to 120 mg/day for breastfeeding women.\(^{32}\) The AIJN\(^{12}\) requires a vitamin C content of at least 200 mg/L in orange juice. This value was well below the vitamin C content found in all samples during frozen storage (Table 2). The vinyl alcohol group present in the ascorbic acid molecule determines its fast-kinetic activity.\(^{14}\) The findings of this work showed that the vitamin C content was always higher than 567.3 mg/L during storage for both BOJ and CBOJ. The initial values of both BOJ (679.8 mg/L) and CBOJ (621.3 mg/L) in the present work were higher than that found by other authors in the juice of Belladonna: a blond cv growing in Calabria (South Italy) (606.7 mg/L).\(^{33}\) Rapisarda et al.\(^{3}\), in four of five studied cultivars, found a slight tendency in vitamin C to decrease during 65 days of orange fruit storage at 6°C. In those five cultivars, the vitamin C content ranged from 550 to 674 mg/L (from 551 to 489 mg/L in cv. Moro), after HPLC analysis. Burdurlu et al.\(^{34}\), after spectrophotometric analysis, found a decreasing trend in vitamin C content in orange juice during 8 weeks’ storage; in addition, they found a negative relation between vitamin C content and increasing storage temperature. Cortés et al.\(^{35}\), with a polarographic system, found that the nutritional quality of orange juice (ascorbic acid) from cv. Navel is maintained longer in pulsed electric field-treated juice than in juice preserved by means of conventional pasteurization treatments.

**Formol number**

The formol number is useful to determine the total amino acidic content in a fruit juice. This parameter is also influenced by the compounds able to fix formaldehyde and at the same time to increase the acidity of a juice fruit.\(^{36}\) During frozen storage, the formol number for both BOJ and CBOJ was always in the range\(^{14–25}\) indicated by the AIJN\(^{12}\) and showed a decreasing trend. After 5 months frozen, it was borderline, i.e. 15.2 for BOJ and 15.1 for CBOJ (Table 2) but above the minimum value indicated by AIJN.\(^{25}\) Esteve et al.\(^{37}\) found a formol number ranging from 19.0 to 22.3 in orange juices after mild pasteurization at 77°C for 20 s and after rapid cooling at 0–2°C. Ramos and Ibarz\(^{38}\) found 1.90 (mg NaOH 0.1 N/100 mL juice) as formol number in concentrated orange juice (60 °Brix).

**Antioxidant activity (DPPH assay)**

Many components contained in orange juice show a quenching activity. As a function of their reaction kinetics with DPPH: they can be divided into three general groups: fast-kinetics, fast + slow-kinetics and slow-kinetics.\(^{14}\) AA value decreased for 3 months during frozen storage and increased...
in the 4th and 5th months’ storage. In BOJ the AA value decreased from 53.39% in T₀ to 42.55% in T₅. A similar trend was found in CBOJ in which the AA value decreased from 39.16% in T₀ to 33.75% in T₅. Findings of other authors were 31.4% in centrifuged and filtered cv. Tarocco juice. Roussos[39] studied the orange juice (C. sinensis) from fruits cultivated on two adjacent Greek commercial organic and integrated orange groves and found 36.8% (organic) and 36.4% (integrated) as AA value in the methanol extract 1:1.

**Total phenols**

The trend in the total phenol content was similar to that of DPPH·, with an initial decrease and a subsequent increase (Table 2). In the BOJ, the phenolic content was 249.5 mg/L at T₀ and decreased to 241.5 and 230.9 mg/L at T₃ and T₄, respectively, but reached a maximum at T₅ (265.7 mg/L). In the CBOJ, the phenolic content was 247.5 mg/L at T₀ and decreased to 235.3 mg/L at T₃ with a subsequent increase to 252 and 258.4 mg/L at T₄ and T₅. Higher phenolic content was found in Sicilian orange juice: 696 mg/L in cv. Moro, 524 mg/L in cv. Tarocco Messina, 507 mg/L in cv. Tarocco Meli, and 571 mg/L in cv. Valencia. [3]

**Total anthocyanins**

Freezing scarcely influenced the total anthocyanins in BOJ, which remained constant throughout all five months of storage (143 mg/L), whereas a significant decrease was found in CBOJ if compared with BOJ. The freezing storage of CBOJ significantly influenced its total anthocyanic content and a constant decrease was found from 129.7 to 122.2 mg/L from T₀ to T₅ (Table 2). This means that the BOJ concentration process caused both a decrease of these precious antioxidants and a juice instability during storage.

**Phenolic acid compounds (HPLC)**

Gallic acid (from the hydroxybenzoic group) was significantly higher in the CBOJ, and in this orange juice formulation it showed a linear increase during frozen storage from 1.73 to 1.91 mg/L at T₅ (Table 3). Three hydroxycinnamic acids were found: chlorogenic, caffeic, and ferulic. Chlorogenic acid is classified for its fast + slow antiradical activity due to its isolated p-catechol group. Chlorogenic acid showed a tendency to increase in both BOJ and CBOJ from T₀ to T₅; from 15.31 to 19.66 mg/L in BOJ and from 12.85 to 27.05 mg/L in CBOJ. Caffeic acid constantly increased up to

<table>
<thead>
<tr>
<th>Table 3. Phenolic acids of BOJ and CBOJ.</th>
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<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>BOJ</td>
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<tr>
<td>T₀</td>
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<tr>
<td>T₁</td>
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<td>T₂</td>
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<td>T₃</td>
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<td>T₄</td>
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<td>T₅</td>
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<tr>
<td>CBOJ</td>
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<td>T₀</td>
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<td>T₃</td>
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<tr>
<td>T₄</td>
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<tr>
<td>T₅</td>
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</table>

Significance *** *** *** ***

Different letters in the same column indicate statistically significant differences.

*** p < 0.001.
the fourth month (52.30% more in BOJ and 39.21% more in CBOJ) and dropped in the last sampling (Table 3). Ferulic acid content increased for the first three months’ frozen storage in both BOJ (0.12–0.27 mg/L) and CBOJ (0.13–0.26 mg/L) and dropped in the fourth and fifth months. Roussos found a similar caffeic acid content in orange juices (C. sinensis, cv. Salustiana) from Greek commercial organic (3.8 mg/mL) and integrated (4.7 mg/mL) orange groves. Arena et al. found a similar ferulic acid content (less than 0.50 mg/L) in BOJ before and after concentration and in orange juice from the market, whereas Destani et al. found 3.61 to 4.28 mg/L ferulic acid in ultrafiltrate BOJ.

**Flavonoid compounds (HPLC)**

Ten flavonoids were identified: two flavonols (rutin and quercetin) and eight flavanones: narirutin, naringin, hesperidin, neoeriocitrin, didymin, eriocitrin, neohesperidin, and hesperetin (Table 4). The rutin content was higher than 1 mg/L only in the first sampling of BOJ, whereas in all other samplings its content ranged from 0.63 to 0.93 mg/L.

The quercetin content was highly significantly different in the 12 samplings (6 for BOJ and 6 for CBOJ) all values were below 0.40 mg/L, with a constant tendency to decrease found in CBOJ. Quercetin is a flavonol commonly present also in other vegetable matrixes: 2.25–15.71 mg/kg in glucuronide form and 4.57–27.26 mg/kg in glucoside form in red wine grape skin; 9.8–90.5 mg/g in different Opuntia species.

Narirutin has slow antiradical activity kinetics; it was the second highest flavonoid in the studied samples. CBOJ showed a higher narirutin content during the storage period; in fact, the lowest narirutin content in CBOJ was in T2 (45.4 mg/L), whereas the highest narirutin content in BOJ was found in T0 (43.0 mg/L). This is lower compared to the narirutin content from frozen orange juices found by Roussos from hand squeezed fruits, and similar to the findings of Galaverna et al. in fresh BOJ or treated in different conditions. Naringin showed a decreasing trend in both BOJ and CBOJ; also this flavanone was higher in the concentrated juice.

Findings on C. sinensis Osbeck emphasize the potential protective effect of hesperidin against ulcers, which is associated with antioxidant and free radical scavenging ability in the stomach mucosal tissue. Under normal conditions, hesperidin molecules are almost insoluble in aqueous solution (15 mg/L) and tend to crystallize quickly. The size of the crystals increases to a certain critical point when they precipitate. In our work, hesperidin was the major flavonoid detected in the studied orange juices. Freezing caused it to decrease; in particular after 5 months’ storage, the hesperidin content dropped by 53.3% in BOJ and 13.20% in CBOJ, even if the lowest hesperidin content of CBOJ (68.7 mg/L) was in T2 and T3, with a calculated 31% drop from the initial content. Roussos found 236 and 261 mg/L hesperidin content in orange juices respectively from organic and integrated orange groves (C. sinensis cv. Salustiana); in this case, the oranges were squeezed and the juice was frozen at −25°C. Galaverna et al. studied a BOJ of cv. Tarocco and found a lower hesperidin content: 45.1 mg/L in fresh juice, 45.5 mg/mL after ultrafiltration permeate treatment, 46.6 mg/mL after reverse osmosis, and 35.2 mg/mL after thermal concentration.

Neoeriocitrin increased remarkably during freezing, from 0.40 to 3.15 mg/L after 5 storage months in BOJ and from 0.70 to 2.25 mg/L after 5 storage months in CBOJ.

Didymin was subject to a constant decrease but with a different trend; in fact, the original didymin content was highest in the CBOJ, and after a 35.48% fall at T2, it increased from 3.09 to 3.56 mg/L from T3 to T5. In the BOJ, the didymin content fell by 83.83% at T2 and remained between 0.52 and 0.86 mg/L. Eriocitrin content decreased during storage in both BOJ and CBOJ and always remained below 0.33 mg/L, in a slightly higher quantity than in frozen, centrifuged, and filtered C. aurantium L. juice. Neohesperidin was always less than 1% but with a twofold higher content in CBOJ. The hesperetin content was always less than 0.52 mg/L, and it was very highly significantly different in the 12 samplings.
**Table 4. Flavonoids of BOJ and CBOJ.**

<table>
<thead>
<tr>
<th></th>
<th>Rutin (mg/L)</th>
<th>Quercetin (mg/L)</th>
<th>Narirutin (mg/L)</th>
<th>Naringin (mg/L)</th>
<th>Hesperidin (mg/L)</th>
<th>Neoeriocitrin (mg/L)</th>
<th>Didymin (mg/L)</th>
<th>Eriocitrin (mg/L)</th>
<th>Neohesperidin (mg/L)</th>
<th>Hesperetin (mg/L)</th>
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<tr>
<td><strong>BOJ</strong></td>
<td></td>
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<tr>
<td>0 T₀</td>
<td>1.01 ± 0.04 a</td>
<td>0.31 ± 0.01 bcd</td>
<td>43.0 ± 0.3 d</td>
<td>2.81 ± 0.08 e</td>
<td>192.2 ± 1.6 a</td>
<td>0.40 ± 0.07 fg</td>
<td>53.2 ± 0.2 a</td>
<td>0.27 ± 0.01 ab</td>
<td>0.40 ± 0.01 c</td>
<td>0.41 ± 0.01 de</td>
</tr>
<tr>
<td>1 T₁</td>
<td>0.87 ± 0.02 bc</td>
<td>0.39 ± 0.05 a</td>
<td>38.6 ± 0.2 e</td>
<td>2.78 ± 0.2 e</td>
<td>121.1 ± 1.3 b</td>
<td>1.01 ± 0.08 e</td>
<td>4.40 ± 0.5 b</td>
<td>0.25 ± 0.01 b</td>
<td>0.41 ± 0.05 c</td>
<td>0.40 ± 0.01 de</td>
</tr>
<tr>
<td>2 T₂</td>
<td>0.72 ± 0.03 de</td>
<td>0.31 ± 0.01 bcd</td>
<td>32.4 ± 3.0 f</td>
<td>1.55 ± 0.07 g</td>
<td>91.1 ± 2.5 e</td>
<td>0.81 ± 0.05 ef</td>
<td>0.86 ± 0.05 d</td>
<td>0.26 ± 0.01 b</td>
<td>0.36 ± 0.01 c</td>
<td>0.41 ± 0.01 de</td>
</tr>
<tr>
<td>3 T₃</td>
<td>0.79 ± 0.08 cd</td>
<td>0.29 ± 0.01 cde</td>
<td>32.1 ± 0.1 f</td>
<td>1.92 ± 0.3 fg</td>
<td>69.2 ± 1.2 f</td>
<td>4.10 ± 0.02 a</td>
<td>0.69 ± 0.2 d</td>
<td>0.27 ± 0.05 ab</td>
<td>0.31 ± 0.04 d</td>
<td>0.45 ± 0.02 bc</td>
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<tr>
<td>4 T₄</td>
<td>0.71 ± 0.01 de</td>
<td>0.29 ± 0.01 cde</td>
<td>30.3 ± 1.2 f</td>
<td>1.52 ± 0.3 gh</td>
<td>88.7 ± 1.9 de</td>
<td>3.80 ± 0.06 ab</td>
<td>0.63 ± 0.05 d</td>
<td>&lt; 0.10</td>
<td>0.36 ± 0.04 cd</td>
<td>0.52 ± 0.02 a</td>
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<tr>
<td>5 T₅</td>
<td>0.63 ± 0.02 e</td>
<td>0.27 ± 0.0 de</td>
<td>32.5 ± 2.1 f</td>
<td>0.70 ± 0.02 h</td>
<td>89.7 ± 3.9 de</td>
<td>3.15 ± 0.2 c</td>
<td>0.52 ± 0.06 d</td>
<td>&lt; 0.10</td>
<td>0.34 ± 0.01 cd</td>
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<td><strong>CBOJ</strong></td>
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<tr>
<td>6 T₀</td>
<td>0.85 ± 0.06 bc</td>
<td>0.36 ± 0.01 ab</td>
<td>56.8 ± 0.7 a</td>
<td>5.75 ± 0.5 ab</td>
<td>100.8 ± 2.8 c</td>
<td>0.70 ± 0.05 ef</td>
<td>5.44 ± 0.3 a</td>
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<td>0.82 ± 0.01 b</td>
<td>0.41 ± 0.01 de</td>
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<tr>
<td>7 T₁</td>
<td>0.86 ± 0.05 bc</td>
<td>0.33 ± 0.03 bc</td>
<td>59.1 ± 1.3 a</td>
<td>6.49 ± 0.4 a</td>
<td>96.4 ± 1.5 cd</td>
<td>0.26 ± 0.1 g</td>
<td>5.12 ± 0.3 a</td>
<td>0.27 ± 0.02 ab</td>
<td>0.76 ± 0.05 b</td>
<td>0.39 ± 0.0 e</td>
</tr>
<tr>
<td>8 T₂</td>
<td>0.89 ± 0.05 abc</td>
<td>0.32 ± 0.01 bcd</td>
<td>45.4 ± 0.5 cd</td>
<td>4.40 ± 0.1 cd</td>
<td>68.87 ± 0.9 f</td>
<td>0.99 ± 0.07 e</td>
<td>3.51 ± 0.03 c</td>
<td>0.27 ± 0.01 ab</td>
<td>0.98 ± 0.02 a</td>
<td>0.39 ± 0.01 de</td>
</tr>
<tr>
<td>9 T₃</td>
<td>0.90 ± 0.05 abc</td>
<td>0.30 ± 0.0 cd</td>
<td>48.8 ± 0.9 bc</td>
<td>4.97 ± 0.1 bc</td>
<td>68.7 ± 1.2 f</td>
<td>3.59 ± 0.4 b</td>
<td>3.09 ± 0.03 c</td>
<td>0.23 ± 0.06 b</td>
<td>0.80 ± 0.01 b</td>
<td>0.45 ± 0.01 b</td>
</tr>
<tr>
<td>10 T₄</td>
<td>0.93 ± 0.03 ab</td>
<td>0.30 ± 0.0 cd</td>
<td>50.6 ± 0.4 b</td>
<td>3.92 ± 0.3 d</td>
<td>85.1 ± 0.1 e</td>
<td>2.57 ± 0.04 d</td>
<td>3.24 ± 0.07 c</td>
<td>&lt; 0.10</td>
<td>0.78 ± 0.02 b</td>
<td>0.42 ± 0.0 cd</td>
</tr>
<tr>
<td>11 T₅</td>
<td>0.89 ± 0.01 abc</td>
<td>0.25 ± 0.04 e</td>
<td>56.9 ± 0.5 a</td>
<td>2.53 ± 0.5 ef</td>
<td>87.5 ± 0.5 e</td>
<td>2.25 ± 0.09 d</td>
<td>3.56 ± 0.2 c</td>
<td>&lt; 0.10</td>
<td>0.77 ± 0.01 b</td>
<td>0.42 ± 0.01 de</td>
</tr>
</tbody>
</table>

**Significance*** *** *** *** *** *** *** *** *** *** **

Different letters in the same column indicate statistically significant differences.

***p < 0.001.
Conclusions

The findings of this work showed that concentration and duration of frozen storage very highly significantly influenced the physicochemical properties of the BOJ. Vitamin C, formol number, naringin, hesperidin, and didymin significantly decreased during frozen storage, whereas chlorogenic and caffeic acid showed an increasing trend in both BOJ and CBOJ. Ten flavonoids were detected: two flavonols (rutin and quercetin) and eight flavanones. Among the latter, hesperidin and narirutin were respectively the first and the second most common. Narirutin, naringin, and neohesperidin were higher in CBOJ than in BOJ. The total anthocyanic content was stable in the frozen BOJ and decreased in the concentrated BOJ after the concentration process, causing anthocyanin instability in the juice.

Funding

This research was supported by Progetto POR CALABRIA FESR 2007/2013- CONSERVO: Nuove Tecnologie per la Valorizzazione della Filiere delle Conserve. ASSE I.

References


