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Physicochemical stability of blood orange juice during frozen storage

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1 **Blood Orange Juice: the Effect of Concentration and Frozen Storage on its**
2 **Physicochemical Properties.**

3
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16
17 *Keywords:* Anthocyanins, Citrus, European Fruit Juice Association, Flavonoids,
18 Freezing, Nutraceuticals, Orange juice, Phenolic acids.

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Summary

Blood orange juice from *Citrus sinensis* (L) Osbek 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 (11.2 – 13.16 g/L). Vitamin C and formol number decreased in both blood orange juice and concentrated blood orange juice during frozen storage, whereas ash content showed an inverse trend. Scavenging abilities of the juices for the DPPH· radical ranged from 35.16% to 75.28% and showed a decreasing trend during the initial 3-4 months and an increase in the last analysed sample. 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, 2 flavonols (rutin and quercetin) and 8 flavanones: narirutin (the second highest flavonoid), naringin, hesperidin (the highest quantity), neoeriocitrin, didymin, eriocitrin, neohesperidin, and hesperitin. Concentration and duration of frozen storage were found to influence the physicochemical properties of blood orange juice in different ways.

INTRODUCTION

South Italy has a tradition of citrus cultivation. In 2011-2012, Italy produced 2,260 tons of orange fruit whereas the largest producers were the USA (7,954.4 tons) and China (6,600 tons) (1). In 2007, Calabria (South Italy) produced 1,213 tons (2) and 1,468 tons in 2011 (3). Citrus juice is normally recognized by consumers for its beneficial effects on human health for its vitamin C content. Orange juice from *Citrus sinensis* (L.) is rich in vitamin C (ascorbic acid) and in other antioxidant compounds. It is one of the most widely recognised functional foods and has been widely studied. Citrus juice induces changes in the human metabolome 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, HDL-c and LDL-c in metabolic syndrome compared to the baseline values. Also improved values of ox-LDL, C-reactive protein and homocysteine levels were found after six months of consumption (6).

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 to maintain its physicochemical properties.

For orange juice producers it is very important to extend all positive properties of blood orange juice (as far as possible) during storage. In order to prevent misleading advertising, the manufacturer should evaluate the shelf life of the product depending on

Comment [LMP1]: Is there more recent statistics? We are in 2017!!!

60 the ingredients used, their concentration and their durability (7). Studies have conducted
61 to improve or extend citrus juice shelf-life by pulsed electric field (8), by ultrafiltration
62 (9), by reverse osmosis and osmotic distillation (10). Beltrán *et al* (11) studied the
63 vitamin C content in mandarin orange (*Citrus reticulata*) juice packed in two different
64 non-transparent cartons made of polyethylene and at different temperatures, and found a
65 common decreasing trend in all samples but with a different rate according to the
66 duration of storage, packaging and temperature.

67 The aim of this work was to study the influence of freezing and frozen storage on the
68 physicochemical properties of blood orange juice (BOJ) and concentrated blood orange
69 juice (CBOJ).

70 MATERIALS AND METHODS

71 Fruit and juice material and analytical criterion

72 Fruits were sampled in Sicily (South Italy). Commercial maturity of fruits was assessed
73 at 10.50 ± 0.80 °Brix (this value was calculated on 50 fruits). The juice used for the
74 experiment was an aliquot randomly sampled from 10 tons of BOJ of *Citrus sinensis*
75 (L.) cv. Moro: a fruit widely grown in Sicily. BOJ was obtained by the following
76 procedure: i, orange collection; ii, orange washing in a tank with water; iii, orange
77 transportation by a conveyor-belt; iv, cutting in two halves; v, squeezing; vi, juice-pulp
78 centrifugation; vii, juice pasteurisation; viii, juice concentration. A SPECIALE
79 equipment model SP40 (Catania, Italy), for citrus juice extraction was used. After
80 squeezing, the obtained juice-pulp mixture was introduced into a horizontal decanter to
81 reduce the pulp content to 2-3% of the total extract. Orange juice was pasteurised at 92
82 °C for 32 sec by a plate pasteuriser. Following this, a SANTORO five-effect falling film
83 evaporator equipment (Catania, Italy) was used to concentrate juice from 10.50 to 55
84 °Brix. The concentration temperature was managed under vacuum from 95 °C in the

Comment [LMP2]: The storage of the juice depends of the fruit quality and the explanation is very weak in this section

Comment [LMP3]: More details are needed about the orchard. Is it from a commercial orchard? The roostock? The climatic conditions? When was the harvest?

85 first effect to 30 °C in the fifth effect, and the pressure changed from 0.74 to 0.30 atm
86 from the first to the fifth effect.

87 Two types of blood orange juice were prepared for the experiment: a BOJ and a
88 concentrated (55 °Brix) blood orange juice (CBOJ). In the laboratory, both BOJ and
89 CBOJ were diluted to 11.2 °Brix as required by AIJN and immediately analysed (T₀).

90 Other aliquots of BOJ and CBOJ (11.2 °Brix) were frozen at -20 °C for 5 months in 100
91 mL polyethylene flasks and analysed monthly (T₁, T₂, T₃, T₄ and T₅). Three samples of
92 each juice were analysed at for each month of storage. In this work, the analyses
93 performed were chosen on the basis of the relevant European regulations, as well as the
94 parameters required by the orange juice industry.

95 Chemicals

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

99 pH

100 This potentiometric analysis was conducted by a pH meter model Crison basic 20 (city, country).

101 °Brix

102 °Brix was determined by a digital refractometer Atago PR-101α (city, country).

103 Titratable Acidity

104 The TA was determined by the IFUMA03 method (12). Two grams of juice and 50 mL
105 of deionized water were placed into a glass beaker and the solution was titrated to a pH
106 of 8.1 with a 0.25 N NaOH solution. The result was expressed as % of citric acid
107 monohydrate.

108 Vitamin C

109 Vitamin C quantification was carried out using an iodometric titration. Ten mL of juice

Comment [LMP4]: What does it mean?
European Fruit Juice Association

110 and 5 mL of deionised water were placed into a beaker. The solution was titrated with a
111 0.01N iodine solution using a 2% starch solution as an indicator. The result is expressed
112 as mg/L of The colour of the orange juice, even if diluted with water, interferes
113 with the iodine-starch system making it difficult to detect the exact point of toning. For
114 this reason the mixture was exposed to an intense light during titration to help
115 distinguish the red colour of the juice from the purple colour of the titrated solution.

116 Formol number

117 The formol number was determined with the method IFUMA30 (12). Ten mL of juice
118 and 10 mL of 40% by volume formaldehyde solution were placed in a beaker and
119 phenolphthalein as the indicator was added. The mixture was titrated with a 0.1 NaOH
120 solution until pH 8.2. The formol number was calculated with the following formula:
121 mL NaOH used for titration/2.

122 Ash

123 An aliquot of sample was evaporated to dryness and ignited at 550 °C to constant
124 weight. Ash was calculated as g/L of juice.

125 **DPPH·** |

126 DPPH· methodology is based on the reduction of the stable free radical 2,2-diphenyl-1-
127 picrylhydrazyl (DPPH·) that strongly absorbs at 515 nm on the corresponding hydrazine
128 by the free radical scavenging activity of the antiradical (13).

129 The antiradical activity of orange juice was carried out using a solution of $6 * 10^{-5}$ M
130 DPPH· in methanol and reading in a spectrophotometer Agilent spectrophotometer
131 model 89090A at 515nm (14).

132 A 2.5 mL aliquot of $6 * 10^{-5}$ M DPPH· in methanol was placed in a quartz cuvette and
133 the absorbance was read at t0 min. At this point 10 µL of juice was placed in the cuvette
134 maintaining a constant agitation for 5 min and the absorbance was read again (t5 min).

Comment [LMP5]: Why not FRAP or ABTS?

135 The result is expressed as % reduction of DPPH· by the following formula:

136
$$\% \text{ DPPH}\cdot = 100 * (1 - (t5 / t0)).$$

137 Total phenols

138 The orange juice was centrifuged at 10,000 rpm for 5 minutes (15) and the supernatant
139 was used to determine the total phenolic content and the antioxidant activity by DPPH·
140 assay. The analysis of total phenols was performed following the colourimetric method
141 of Folin-Ciocalteu. Into a 25 mL glass flask were placed: 0.2 mL juice, 5 mL deionised
142 water and 1 mL Folin-Ciocalteu reagent. After 8 minutes, 10 mL of 20% Na₂CO₃
143 solution was added and the mixture was made up to volume with deionised water. At
144 the same time a reference solution (blank) was prepared using the same amount of
145 reagents but without the sample. The mixtures were left in the dark for two hours before
146 reading the absorbance at 765nm in a Agilent spectrophotometer model 89090A ([city,](#)
147 [country](#)). The total phenol content was expressed as mg of gallic acid /L of juice.

148 Total anthocyanic content.

149 Anthocyanins were analysed using the pH differential method No 2 suggested by
150 Rapisarda et al (16). Results were expressed as mg/L of cyaniding 3-glucoside chloride.
151 Spectrophotometric analysis was conducted using a Perkin Elmer instrument model
152 Lambda 2 ([city, country](#)):-

153 Phenolic acids and Flavonoids

154 The orange juice was centrifuged at 10,000 rpm (5 min) and filtered through a 0.45 µM
155 particle size and 25 mm diameter filter (Chromafil RC-45/25, Macherey-Nagel GmbH
156 & Co. KG) before injection (20 µL) directly into the HPLC (15-17).

157 The HPLC analysis was carried out using a Knauer instrument equipped with a DAD
158 detector (model 2600) ([city, country](#)); the selected wavelengths were 254 nm for
159 quercetin and rutin; 280 nm for gallic acid, eriocitrin, neoeriocitrin, narirutin, naringin,

160 hesperidin, neohesperidin, didymin and hesperetin; and 305 nm for chlorogenic acid,
161 caffeic acid and ferulic acid. For quercetin and rutin, preliminary tests were conducted
162 at 254, 280, 305 and 360 nm, and the maximum absorbance was found at 254 nm, this
163 was in accordance with studies of other authors (18, 19, 20, 21, 22). Flavonoids have a
164 maximum absorbance at 254 nm and at 360 nm. HPLC-DAD is commonly used in the
165 study of antioxidants in other matrices as grape berry skin (23).

166 Elution was with a binary gradient: bi-deionized water (mobile phase A) and acetonitrile
167 (mobile phase B), both mobile phases were acidified (pH 3) with formic acid. Analyses
168 were performed using the following gradient: 95% A and 5% B in isocratic (20 min);
169 eluent B increased from 5 to 40% from 20 min to 50 min; from 40% to 95% from 50 to
170 60 min; and then decreased from 95% to 5% from 60 to 65 min and finally 95% A and
171 5% B in isocratic from 65 to 70 min at 1 mL/min flow rate. The separation column was
172 a Knauer C18 Eurosphere II with pre-column (250 mm length x 4.6 mm internal
173 diameter x 5 µm particle size). Phenolic compounds were quantified with the external
174 standard technique and using a standard solution containing all the detected phenols.

175 HPLC method validation

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

180 *Linearity.* The combined standard solutions were prepared at five different
181 concentrations and injected into the chromatographic system. The correlation
182 coefficient was calculated in the range of 0.9970-0.9981 for phenolic acids and in the
183 range of 0.9984-0.9989 for flavonoids.

184 *Limits of detection and quantification.* The limit of detection (LOD) varied from 0.01 to

185 0.03 mg/L for phenolic acids and between 0.01 and 0.02 mg/L for flavonoids. The limit
186 of quantification (LOQ) varied from 0.03 to 0.05 mg/L for both phenolic acids and
187 flavonoids.

188 Statistical analysis

189 Analyses were conducted in triplicate; means and standard deviations were calculated
190 by the Excel software (2007 version). One way ANOVA was applied to the different
191 storing date using SPSS version 15.0 for Windows (SPSS Inc., Chicago IL, U.S.A.);
192 differences between sample means were analysed by Tukey's method of multiple
193 comparison at $p < 0.05$.

194 RESULTS AND DISCUSSION

195 pH

196 pH showed a slight tendency to decrease for both BOJ and CBOJ during frozen storage
197 (Table 2). The highest values were found in T₀ (3.74 and 3.81 respectively for BOJ and
198 CBOJ). After 5 months' storage, pH values decreased to 3.34 (BOJ) and 3.50 (CBOJ),
199 (Table 2). Similar data were found in cv. Navel (3.7), in Tarocco cv. (3.6), in Moro cv.
200 (3.6) and in Sanguinello cv (3.6) from Sicily (24). Other Authors found similar values
201 with a slight increase from 3.34 to 3.43 in the pH value in non-processed orange juice
202 during 12 days storage at 4 °C (25).

203 °Brix

204 This measure represents the level of total soluble solids in a given sample, the system
205 previously having been calibrated with sucrose solutions at known concentration.

206 °Brix was similar in both BOJ and CBOJ during five months of storage and ranged from
207 10.7 to 11.5 (Table 1). In all cases Brix values were above 10.0 (Table 1): the minimum
208 indicated by the European Fruit Juice Association (26). Destani *et al* (27) found 11.0
209 °Brix in squeezed, depectinized and filtered blood orange juice cv. Tarocco from

210 Calabria (South Italy). In squeezed and centrifuged orange juices, Ingallinera *et al* (24)
211 found a 13.4 °Brix in cv. Navel, 13.5 °Brix in cv. Tarocco, 11.6 °Brix in cv. Moro and
212 12.6 °Brix in cv. Sanguinello from Sicily (South Italy). Other Authors found lower
213 °Brix values in non-sonicated (8.9 °Brix) and sonicated (8.8-8.9 °Brix) fresh filtered
214 orange juice of *Citrus sinensis* cv. Valencia from Spain (28).

215 Titratable Acidity

216 Acidity increased in the BOJ over the five months' storage, from 12.66 in T₀ to 13.16
217 g/L in T₅ (0.5 acidity more, i.e. 3.94% increase). Similar results were found in CBOJ,
218 from 11.17 g/L in T₀ to 11.60 g/L in T₅ (0.7 g/L acidity more, i.e. 6.25% increase),
219 (Table 2). All values were in the range indicated by the AIJN (26), 5.8-15.4 g/L (Table
220 1). The titratable acidity evolution found in the studied samples confirmed the pH
221 decrease, the higher the acidity, the lower the pH value. Tiwari *et al* (28), in squeezed,
222 filtered and sonicated juice of *Citrus sinensis* cv. Valencia from Spain, found 6.3 g/L as
223 a titratable acidity, half of that in our samples. Kelebek *et al* (29), in squeezed and
224 centrifuged blood orange juice, found 11.3 and 13.4 g/L as TA after freezing at -18 °C
225 (cvs Moro and Sanguinello respectively). Palma *et al* (30) found 13.8 g/L in Tarocco cv
226 juice from South Sardinia (Italy); they also found a slight decrease in TA content after
227 hot water postharvest treatments (from 20 °C to 59 °C) and after simulated quarantine
228 conditions for fruit disinfestation. In Moro cv juice, Lo Scalzo *et al*. (31) found an effect
229 in TA content caused by thermal treatments, they found 106 meq/100g in untreated
230 orange juice; 80.1 meq/100g in squeezed segments previously blanched for 6 min at 80
231 °C; 101 meq/100g in squeezed segments pasteurised at 80 °C for 1 min.

232 Vitamin C

233 Vitamin C is found almost exclusively in foods of plant origin. Its absorption in human
234 body occurs in the buccal mucosa, stomach and small intestine. Buccal absorption is

235 thought to be by way of passive diffusion through the membrane of the buccal mucosal
236 cells. Gastrointestinal absorption of the vitamin is rapid and efficient, and an active
237 carrier-mediated transport system has been suggested, especially at low concentrations
238 (32). Vitamin C is one of the most important parameters used by consumers when a
239 nutraceutical is chosen. In oranges the predominant form of vitamin C is ascorbic acid
240 whereas dehydroascorbic acid is less than 10% of total vitamin C (33). It is a water-
241 soluble molecule that cannot be produced by humans but has to be ingested in the diet.
242 The National Institute of Health recommends a daily intake of 15 mg/day for 1-3 year
243 old children up to 120 mg/day for breastfeeding women (34). The AIJN (26) requires a
244 vitamin C content of at least 200 mg/L in orange juice. This value was largely below the
245 vitamin C content found in all samples during frozen storage (Table 2). The vinyl
246 alcohol group present in the ascorbic acid molecule determines its fast-kinetic activity.
247 The findings of this work showed that the vitamin C content was always higher than
248 560 mg/L during four months storage for both BOJ and CBOJ, but a fall was found in
249 the fifth month in both BOJ and CBOJ. The initial value of both BOJ (679.8 mg/L) and
250 CBOJ (621.3 mg/L) in the present work were higher than that found by other authors in
251 the juice of Belladonna: a blond cv growing in Calabria (South Italy) (606.7 mg/L) (35).
252 Rapisarda *et al* (33), in four of five studied cultivars, found a slight tendency in vitamin
253 C to decrease during 65 days storage of orange fruits at 6 °C. In those five cultivars, the
254 vitamin C content ranged from 550 to 674 mg/L, (from 551 to 489 mg/L in cv Moro),
255 after HPLC analysis. Burdurlu *et al* (36), after spectrophotometric analysis found a
256 decreasing trend in vitamin C content in orange juice during 8 weeks' storage; in
257 addition they found a negative relation between vitamin C content and increasing
258 storage temperature. Cortés *et al* (37), with a polarographic system found that the
259 nutritional quality of orange juice (ascorbic acid) from Navel cv is maintained longer in

260 pulsed electric field-treated juice than in juice preserved by means of conventional
261 pasteurization treatments.

262 Formol number

263 The formol number is useful to determine the total aminoacidic content in a fruit juice.
264 This parameter is also influenced by the compounds able to fix formaldehyde and at the
265 same time to increase the acidity of a juice fruit.

266 During frozen storage the formol number for both BOJ and CBOJ was always in the
267 range (15-26) indicated by the AIJN (26) and showed a decreasing trend. After 5
268 months frozen it was borderline i.e. 15.2 for BOJ and 15.1 for CBOJ (Table 2) but
269 above the minimum value indicated by AIJN (26). Esteve *et al* (38) found a formol
270 number ranging from 19.0 to 22.3 in orange juices after mild pasteurization at 77 °C for
271 20 seconds and after rapid cooling at 0-2 °C. Ramos and Ibarz (39) found 1.90 as
272 formol number in concentrated orange juice (60 °Brix).

273 Ash

274 Ash content increased during frozen storage. In BOJ the ash content was 1.78 g/L at T₀
275 and it was 5.16 g/L at T₅ (i.e. 2.90 times higher). In CBOJ the ash content was 1.81 at
276 T₀ and it was 3.89 g/L (i.e. 2.20 times higher) after 5 months' storage. A lower ash
277 content (1.2 g/L) was found by Chaudhary and Verma in an orange juice (40). Corpas *et*
278 *al* (41) found 3.33 g/L ash in fresh and untreated orange juice obtained from squeezed
279 oranges from a Romanian hypermarket. Grigelmo-Miguel and Martín-Belloso (42)
280 found 2.8%, 2.6% and 3.1% ash content in dietary fibers from *Citrus sinensis* cv Navel,
281 Salustiana and Valencia late, respectively.

282 DPPH·

Comment [LMP6]: Why is higer? Could you explain, please?

283 Many components contained in orange juice show a quenching activity. As a function of
284 their reaction kinetics with DPPH· they can be divided into three general groups: fast-
285 kinetics, fast + slow-kinetics and slow-kinetics (13).

286 DPPH· value decreased for three months during frozen storage and increased in the
287 fourth and fifth months' storage. In BOJ the DPPH· value decreased from 53.39% in T₀
288 to 43.61% in T₂ and increased to 75.28% and 73.21% in T₄ and T₅ respectively. A
289 similar trend was found in CBOJ in which the DPPH· value decreased from 39.16% in
290 T₀ to 35.16% in T₂ and increased to 69.84% and 65.08% in T₄ and T₅. A similar trend
291 was found in the total phenol content. Findings of other Authors were 31.4% in
292 centrifuged and filtered Tarocco cv juice (30). Roussos (43) studied the orange juice
293 (*Citrus sinensis*) from fruits cultivated on two adjacent Greek commercial organic and
294 integrated orange groves and found 36.8% (organic) and 36.4% (integrated) as DPPH·
295 value in the methanol extract 1:1.

296 Total phenols

297 The trend in the total phenol content was similar to that of DPPH·, with an initial
298 decrease and a subsequent increase (Table 2). In the BOJ the phenolic content was
299 249.5 mg/L at T₀ and decreased to 241.5 mg/L and 230.9 mg/L at T₃ and T₄ respectively
300 but reached a maximum at T₅ (265.7 mg/L). In the CBOJ the phenolic content was
301 247.5 mg/L at T₀ and decreased to 235.3 mg/L at T₃ with a subsequent increase to 252.
302 mg/L and 258.4 mg/L at T₄ and T₅. Higher phenolic content was found in Sicilian
303 orange juice: 696 mg/L in cv Moro, 524 mg/L in cv Tarocco, 507 mg/L in cv Tarocco
304 and 571 mg/L in cv Valencia (33).

305 Total anthocyanins

306 Freezing scarcely influenced the total anthocyanins in BOJ which remained constant
307 throughout all five months of storage (143 mg/L), whereas a significant decrease was

308 found in CBOJ if compared with BOJ. The freezing storage of CBOJ significantly
309 influenced its total anthocyanic content and a constant decrease was found from 129.7
310 to 122.2 mg/L from T₀ to T₅ (Table 2). This means that the BOJ concentration process
311 caused both a decrease of these precious antioxidants and a juice instability during
312 storage.

313 Phenolic acids (HPLC)

314 Gallic acid (from the hydroxybenzoic group) was significantly higher in the CBOJ and
315 in this orange juice formulation it showed a linear increase during frozen storage from
316 1.73 mg/L to 1.91 mg/L at T₅ (Table 3).

317 Three hydroxycinnamic acids were found: chlorogenic, caffeic and ferulic. Chlorogenic
318 acid is classified for its fast + slow antiradical activity due to their isolated *p*-catechol
319 group (44). Chlorogenic acid showed a tendency to increase in both BOJ and CBOJ
320 from T₀ to T₅: from 15.31 to 19.66 mg/L in BOJ and from 12.85 to 27.05 mg/L in
321 CBOJ. Caffeic acid constantly increased up to the fourth month (52.30% more in BOJ
322 and 39.21% more in CBOJ) and dropped in the last sampling (Table 3). Ferulic acid
323 content increased for the first three months' frozen storage in both BOJ (0.12 – 0.27
324 mg/L) and CBOJ (0.13 – 0.26 mg/L) and dropped in the fourth and fifth month. Roussos
325 (43) found a similar caffeic acid content in orange juices (*Citrus sinensis*, cv Salustiana)
326 from Greek commercial organic (3.8 mg/mL) and integrated (4.7 mg/mL) orange
327 groves. Arena *et al.* (44) found a similar ferulic acid content (less than 0.50 mg/L) in
328 blood orange juice before and after concentration and in orange juice from the market,
329 whereas Destani *et al* (27) found 3.61 to 4.28 mg/L ferulic acid in ultra filtrate blood
330 orange juice.

331 Flavonoids (HPLC)

332 Ten flavonoids were identified: 2 flavonols (rutin and quercetin) and 8 flavanones:
333 narirutin, naringin, hesperidin, neoeriocitrin, didymin, eriocitrin, neohesperidin, and
334 hesperitin (Table 4).

335 The rutin content was higher than 1 mg/L only in the first sampling of BOJ, whereas in
336 all other samplings its content ranged from 0.63 to 0.93 mg/L.

337 The quercetin content was highly significantly different in the twelve samplings (6 for
338 BOJ and 6 for CBOJ) all values were below 0.40 mg/L, with a constant tendency to
339 decrease found in CBOJ. Quercetin is a flavonol commonly present also in other
340 vegetable matrixes: 2.25-15.71 mg/kg in glucuronide form and 4.57-27.26 mg/kg in
341 glucoside form in red wine grape skin (23); 9.8-90.5 mg/g in different *Opuntia* species
342 (45).

343 Narirutin has slow antiradical activity kinetics (46); it was the second- major flavonoid
344 in the studied samples. CBOJ showed a higher narirutin content during the storage
345 period, in fact, the lowest narirutin content in CBOJ was in T₂ (45.4 mg/L), whereas the
346 highest narirutin content in BOJ was found in T₀ (43.0 mg/L), in a lower amount
347 compared to the narirutin content from frozen orange juices found by Roussos (43) from
348 hand squeezed fruits, and in a similar content to findings of Galaverna *et al* (10) in fresh
349 blood orange juice or treated in different conditions.

350 Naringin showed a decreasing trend in both BOJ and CBOJ, also this flavanone was
351 higher in the concentrated juice.

352 Findings on *Citrus sinensis* (L.) Osbeck emphasise the potential protective effect of
353 hesperidin against ulcers which is associated with antioxidant and free radical
354 scavenging ability in the stomach mucosal tissue (47). Under normal conditions,
355 hesperidin molecules are almost insoluble in aqueous solution (15 mg/L), and tend to
356 crystallize quickly. The size of the crystals increases to a certain critical point when they

357 precipitate (48). In our work, hesperidin was the major flavonoid detected in the studied
358 orange juices. Freezing caused it to decrease, in particular after 5 months' storage, the
359 hesperidin content dropped by 53.3% in BOJ and 13.20% in CBOJ, even if the lowest
360 hesperidin content of CBOJ (68.7 mg/L) was in T₂ and T₃, with a calculated 31% drop
361 from the initial content. Roussos (43) found 236 and 261 mg/L hesperidin content in
362 orange juices respectively from organic and integrated orange groves (*Citrus sinensis* cv
363 Salustiana), in this case the oranges were squeezed and the juice was frozen at -25 °C.
364 Galaverna *et al* (10) studied a blood orange juice of cv Tarocco and found a lower
365 hesperidin content: 45.1 mg/L in fresh juice; 45.5 mg/mL after ultrafiltration permeate
366 treatment; 46.6 mg/mL after reverse osmosis and 35.2 mg/mL after thermal
367 concentration.

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

370 Didymin was subjected to a constant decrease but with a different trend, in fact, the
371 original didymin content was highest in the CBOJ and after a 35.48% fall at T₂, it
372 increased from 3.09 to 3.56 mg/L from T₃ to T₅. In the BOJ the didymin content fell by
373 83.83% at T₂ and remained between 0.52 and 0.86 mg/L.

374 Eriocitrin content decreased during storage in both BOJ and CBOJ and always remained
375 below 0.33 mg/L, in a slightly by higher quantity than in frozen, centrifuged and filtered
376 *Citrus aurantium* L. juice (49).

377 Neohesperidin was always less than 1% but with a two-fold higher content in CBOJ.

378 The hesperetin content was always less than 0.52 mg/L and it was very highly
379 significantly different in the 12 samplings. BOJ and CBOJ showed a neohesperidin
380 content at least 10 times higher than in other citrus juice as *Poncirus trifoliata* (50).

381

CONCLUSIONS

382 The findings of this work showed that concentration and duration of frozen storage very
383 highly significantly influenced the physicochemical properties of the blood orange
384 juice. Vitamin C, formol number, naringin, hesperidin, and didymin significantly
385 decreased during frozen storage, whereas chlorogenic and caffeic acid showed an
386 increasing trend in both BOJ and CBOJ. Ten flavonoids were detected: 2 flavonols
387 (rutin and quercetin) and 8 flavanones. Among the latter, hesperidin and narirutin were
388 respectively the first and the second most represented. Narirutin, naringin and
389 neohesperidin were higher in CBOJ than in BOJ. The total anthocyanic content was
390 stable in the frozen blood orange juice and decreased in the concentrated blood orange
391 juice after the concentration process, caused anthocyanin instability in the juice.

Comment [LMP7]: And the color? One of the most important parameters for consumers is the juice color

392 REFERENCES

- 393 1. FAO, 2012 - Food and Agriculture Organization of the United Nations. Citrus fruit
394 fresh and processed, annual statistic **2012**.
395 [http://www.fao.org/fileadmin/templates/est/COMM_MARKETS_MONITORING](http://www.fao.org/fileadmin/templates/est/COMM_MARKETS_MONITORING/Citrus/Documents/CITRUS_BULLETIN_2012.pdf)
396 [/Citrus/Documents/CITRUS_BULLETIN_2012.pdf](http://www.fao.org/fileadmin/templates/est/COMM_MARKETS_MONITORING/Citrus/Documents/CITRUS_BULLETIN_2012.pdf) (accessed on November,
397 2016).
- 398 2. Proto, A.R.; Zimbalatti, G. Risk Assessment of repetitive movements in the citrus
399 fruit industry. *J. Agric. Saf. Health.* **2010**,*16*,219-228.
- 400 3. Istat (**2016**). http://dati.istat.it/Index.aspx?DataSetCode=DCSP_COLTIVAZ&Lang=it
- 401 4. Medina, S.; Ferreres, F.; Garcia-Viguera, C.; Horcajada, M.N; Orduna, J.; Saviron,
402 M.; Zurek, G.; Martinez-Sanz, Jm.; Gil, J.I.; Gil-Izquierdo, A. Non-targeted
403 metabolomic approach reveals urinary metabolites linked to steroid biosynthesis
404 pathway after ingestion of citrus juice. *Food Chem.* **2013**,*136*,938-946.
405 <http://dx.doi.org/10.1016/j.foodchem.2012.09.004>

- 406 5. Sánchez-Moreno, C.; Cano, M.P.; de Ancos, B.; Plaza, L.; Olmedilla, B.; Granada,
407 F.; Martín, A. Effect of orange juice intake on vitamin C concentrations and
408 biomarkers of antioxidant status in humans. *Am. J. Clin. Nutr.* **2003**,*78*,454-460.
- 409 6. Mulero J, Bernabé J, Cerdá B, García-Viguera C, Moreno DA, Albaladejo MD,
410 Avilés, F, Parra S, Abellán J, Zafrilla P. Variations on cardiovascular risk factors
411 in metabolic syndrome after consume of a citrus-based juice. *Clin. Nutr.*
412 **2012**,*31*,372-377.
- 413 7. Scordino, M.; Sabatino, L.; Lazzaro, F.; Borzi, M.A.; Gargano, M.; Traulo, P.;
414 Gagliano, G. Blood Orange Anthocyanins in fruit beverages: how the commercial
415 shelf life reflects the quality parameter. *Beverages* **2015**,*1*,82-94.
416 DOI:10.3390/beverages1020082
- 417 8. Cserhalmi, Zs.; Sass-Kiss, A.; Tóth-Markus, M.; Lechner, N. Study of pulsed electric
418 field treated citrus juices. *Innov. Food Sci. Emerg.* **2006**,*7*,49-54.
419 DOI:10.1016/j.ifset.2005.07.001
- 420 9. Cassano, A.; Marchio, M.; Orioli, E. Clarification of blood orange juice by
421 ultrafiltration: analyses of operating parameters, membrane fouling and juice
422 quality. *Desalination* **2007**,*212*,15-27. DOI:10.1016/j.desal.2006.08.013
- 423 10. Galaverna, G.; Di Silvestro, G.; Cassano, A.; Sforza, S.; Dossena, A.; Drioli, E.;
424 Marchelli, R. A new integrated membrane process for the production of
425 concentrated blood orange juice: Effect on bioactive compounds and antioxidant
426 activity. *Food Chem.* **2008**,*106*,1021-1030. DOI:10.1016/j.foodchem.2007.07.018
- 427 11. Beltrán, F.; Pérez-López, A.J.; López-Nicolás, J.M.; Carbonell-Barrachina, A.A.
428 Color and vitamin C content in mandarin orange juice as affected by packaging
429 material and storage temperature. *J. Food Process. Pres.* **2009**,*33*,27-40. DOI:
430 10.1111/j.1745-4549.2008.00247.x

- 431 12. IFUMA (www.ifu-fruitjuice.com/ifu-methods)
- 432 13. Sendra, J.M.; Sentandreu, E.; Navarro, J.L. Reduction kinetics of the free stable
433 radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) for determination of the antiradical
434 activity of citrus juices. Eur. Food Res. Technol. **2006**,223,615-624. DOI
435 10.1007/s00217-005-0243-3
- 436 14. Kalantzakis, G.; Blekas, G.; Pegklidou, K.; Boskou, D. Stability and radical-
437 scavenging activity of heated olive oil and other vegetable oils. Eur. J. Lipid Sci.
438 Tech. **2006**,108,329-35. DOI 10.1002/ejlt.200500314
- 439 15. González-Molina, E.; Moreno, D.A.; García-Viguera, C. Comparison of ‘Verna’
440 lemon juice quality for new ingredients and food products. Sci Hortic-Amsterdam.
441 **2009**,120,353-59. DOI:10.1016/j.scienta.2008.11.010
- 442 16. Rapisarda, P.; Fanella, F.; Maccarone, E. Reliability of analytical method for
443 determining anthocyanins in blood orange juice. J. Agric. Food Chem.
444 **2000**,48,2249-2252.
- 445 17. Klimczak, I.; Malecka, M.; Szlachta, M.; Gliszczynska-Swiglo, A. Effect of storage
446 on the content of polyphenols, vitamin C and the antioxidant activity of orange
447 juices. J. Food Compos. Anal. **2007**,20,313-322. DOI:10.1016/j.jfca.2006.02.012
- 448 18. Sladkovský, R.; Solich, P.; Opletal, L. Simultaneous determination of quercetin,
449 kaempferol and (E)-cinnamic acid in vegetative organs of *Schisandra chinensis*
450 Baill. by HPLC. J. Pharmaceut. Biomed. **2001**,24,1049–1054
- 451 19. Zu, Y.; Li, C.; Fu, Y.; Zhao, C. Simultaneous determination of catechin, rutin,
452 quercetin, kaempferol and isorhamnetin in the extract of sea buckthorn
453 (*Hippophae rhamnoides* L.) leaves by RP-HPLC with DAD. J. Pharmaceut.
454 Biomed. **2006**,41,714-719. DOI: 10.1016/j.jpba.2005.04.052

- 455 20. Granica, S.; Krupa, K.; Kiebowska, A.; Kiss, A.K. Development and validation of
456 HPLC-DAD-CAD-MS3 method for qualitative and quantitative standardization
457 of polyphenols in *Agrimoniae eupatoriae herba* (Ph. Eur). J. Pharmaceut.
458 Biomed. **2013**,*86*,112–22. <http://dx.doi.org/10.1016/j.jpba.2013.08.006>
- 459 21. Jia, W.; Wang, C.; Wang, Y.; Pan, G.; Jiang, M.; Li, Z.; Zhu, Y. Qualitative and
460 Quantitative Analysis of the Major Constituents in Chinese Medical Preparation
461 Lianhua-Qingwen Capsule by UPLC-DAD-QTOF-MS. Scientific World J. **2015**, 1-19.
462 <http://dx.doi.org/10.1155/2015/731765>
- 463 22. Kicel, A.; Owczareka, A.; Michela, P.; Skalicka-Woźniakb, K.; Kissc, A.K.;
464 Olszewska, M.A. Application of HPLC, UHPLC-PDA-ESI-MS³ and HPLC-
465 PDA methods for rapid, one-step preparative separation and quantification of rutin
466 in Forsythia flowers. Ind. Crop Prod. **2015**,*76*,86–94.
467 <http://dx.doi.org/10.1016/j.indcrop.2015.06.019>
- 468 23. Giuffrè, A.M. HPLC-DAD detection of changes in phenol content of red berry skins
469 during grape ripening. Eur. Food Res. Technol. **2013**,*327*,555-564. DOI
470 10.1007/s00217-013-2033-7
- 471 24. Ingallinera, B.; Barbagallo, R.N.; Spagna, G.; Palmeri, R.; Todaro, A. Effects of
472 thermal treatments on pectinesterase activity determined in blood oranges juices.
473 Enzyme Microb. Tech. **2005**,*36*:258-263. DOI:10.1016/j.enzmictec.2004.08.041
- 474 25. Del Caro, A.; Piga, A.; Vacca, V.; Agabbio, M. Changes of flavonoids, vitamin C
475 and antioxidant capacity in minimally processed citrus segments and juices during
476 storage. Food Chem. **2004**,*84*,99-105. DOI:10.1016/S0308-8146(03)00180-8
- 477 26. AIJN (**2016**). <http://www.aijn.org>
- 478 27. Destani, F.; Cassano, A.; Fazio, A.; Vincken, J.P.; Gabriele, B. Recovery and
479 concentration of phenolic compounds in blood orange juice by membrane

- 480 operations. *Journal Food Eng.* **2013**,*117*,263-71.
481 <http://dx.doi.org/10.1016/j.jfoodeng.2013.03.001>
- 482 28. Tiwari, B.K.; Muthukumarappan, K.; O'Donnell, C.P.; Cullen, P.J. Colour
483 degradation and quality parameters of sonicated orange juice using response
484 surface methodology. *LWT - Food Sci. Technol. – Leb.* **2008**,*41*,1876-1883.
485 DOI:10.1016/j.lwt.2007.11.016
- 486 29. Kelebek, H.; Canbas, A.; Selli, S. Determination of phenolic composition and
487 antioxidant capacity of blood orange juices obtained from cvs. Moro and
488 Sanguinello (*Citrus sinensis* (L.) Osbeck) grown in Turkey. *Food Chem.*
489 **2008**,*107*,1710-1716.DOI:10.1016/j.foodchem.2007.10.004
- 490 30. Palma, A.; D'Aquino, S., Vanadia, S.; Angioni, A.; Schirra, M. Cold quarantine
491 responses of 'Tarocco' oranges to short hot water and thiabendazole postharvest
492 dip treatments. *Postharvest Biol. Tec.* **2013**,*78*,24–33.
493 <http://dx.doi.org/10.1016/j.postharvbio.2012.12.002>
- 494 31. Lo Scalzo, R.; Iannoccaria, T.; Summa, C.; Morelli, R, Rapisarda, P. Effect of
495 thermal treatments on antioxidant and antiradical activity of blood orange juice.
496 *Food Chem.* **2004**,*85*,41-47. DOI:10.1016/j.foodchem.2003.05.005
- 497 32. Basu, T.K.; Donaldson, D. Intestinal absorption in health and disease:
498 micronutrients. *Best Pract. Res. Cl. Ha.* **2003**,*17*,957-979.
- 499 33. Rapisarda, P.; Lo Bianco, M.; Pannuzzo, P.; Timpanaro, N. Effect of cold storage on
500 vitamin C, phenolics and antioxidant activity of five orange genotypes (*Citrus*
501 *sinensis* (L.) Osbeck). *Postharvest Biol. Tec.* **2008**,*49*,348-354.
502 DOI:10.1016/j.postharvbio.2008.02.002
- 503 34. NIH **2015**. <https://ods.od.nih.gov/factsheets/VitaminC-Consumer/>. Accessed on 15th
504 December 2015

- 505 35. Sicari, V.; Dorato, G.; Giuffrè, A.M.; Rizzo, P.; Albuina, A.R. The effect of different
506 packaging on physical and chemical properties of oranges during storage. *J. Food*
507 *Process. Pres.* (**in press**). DOI 10.1111/jfpp.13168
- 508 36. Burdurlu, H.S.; Koca, N.; Karadeniz, F. Degradation of vitamin C in citrus juice
509 concentrates during storage. *Journal Food Eng.* **2006**,74,211–216.
510 DOI:10.1016/j.jfoodeng.2005.03.026
- 511 37. Cortés, C.; Esteve, M.J.; Frígola, A. Effect of refrigerated storage on ascorbic acid
512 content of orange juice treated by pulsed electric fields and thermal pasteurization.
513 *Eur. Food Res. Technol.* **2008**,227,629-635. DOI 10.1007/s00217-007-0766-x
- 514 38. Esteve, M.J.; Frígola, A.; Rodrigo, C.; Rodrigo, D. Effect of storage period under variable
515 conditions on the chemical and physical composition and colour of Spanish refrigerated
516 orange juices. *Food Chem. Toxicol.* **2005**,43,1413-1422. DOI:10.1016/j.fct.2005.03.016
- 517 39. Ramos, A.M.; Ibarz, A. Density of juice and fruit puree as a function of soluble
518 solids content and temperature. *Journal Food Eng.* **1998**,35,57-63.
- 519 40. Chaudhary, M.; Verma, S.K. Analysis of the physicochemical properties of the
520 processed fruits and vegetable products. *International Journal of Pharma and Bio*
521 *Sciences* **2011**,2,B660-B666.
- 522 41. Corpas, L.; Velcirov, A.B.; Ravis, A.; Olariu, L.; Graviã, C.; Armadi, M. Physico-
523 chemical characterization of some fruits juices from Romanian hypermarket
524 fruits. *J. Agroaliment. Proc. Technol.* **2012**,18,95-99.
- 525 42. Grigelmo-Miguel, N.; Martín-Belloso, O. Characterization of dietary fiber from
526 orange juice extraction. *Food Res. Int.* **1999**,31,355-361.
- 527 43. Roussos, P.A. Phytochemicals and antioxidant capacity of orange (*Citrus sinensis*
528 (L.) Osbeck cv. Salustiana) juice produced under organic and integrated farming

- 529 system in Greece. *Sci. Hortic-Amsterdam* **2011**,*129*,253-258.
530 DOI:10.1016/j.scienta.2011.03.040
- 531 44. Arena, E.; Fallico, B.; Maccarone, E. Evaluation of antioxidant capacity of blood
532 orange juices as influenced by constituents, concentration process and storage.
533 *Food Chem.* **2001**,*74*,423-427.
- 534 45. Kuti, J.O. Antioxidant compounds from four *Opuntia* cactus pear fruit varieties.
535 *Food Chem.* **2004**,*85*,527-533. DOI:10.1016/S0308-8146(03)00184-5
- 536 46. Betoret, E.; Sentandreu, E.; Betoret, N.; Fito, P. Homogenization pressures applied
537 to citrus juice manufacturing. Functional properties and application. *Journal Food*
538 *Eng.* **2012**,*111*,28-33. DOI:10.1016/j.jfoodeng.2012.01.035
- 539 47. Bigoniya, P.; Singh, K. Ulcer protective potential of standardized hesperidin, a
540 citrus flavonoid isolated from *Citrus sinensis*. *Rev. Bras. Farmacogn.*
541 **2014**,*24*,330-40. <http://dx.doi.org/10.1016/j.bjp.2014.07.011>
- 542 48. Ben-Shalom, N.; Pinto, R. Natural colloidal particles: the mechanism of the specific
543 interaction between hesperidin and pectin. *Carbohydr. Polym.* **1999**,*38*,179-182.
- 544 49. Barreca, D.; Bellocco, E.; Caristi, C.; Leuzzi, U.; Gattuso, G. Distribution of C- and
545 O-glycosyl flavonoids, (3-hydroxy-3-methylglutaryl) glycosyl flavanones and
546 furocoumarins in *Citrus aurantium* L. juice. *Food Chem.* **2011**,*124*,576-582.
547 DOI:10.1016/j.foodchem.2010.06.076
- 548 50. Tundis, R.; Bonesi, M.; Sicari, V.; Pellicanò, T.M.; Tenuta, M.C.; Leporini M.;
549 Manichini F, Loizzo, M.R. *Poncirus trifoliata* (L.) Raf.: Chemical composition,
550 antioxidant properties and hypoglycaemic activity via the inhibition of α -amylase and
551 α -glucosidase enzymes. *J. Funct. Foods.* **2016**,*5*,477-485.
552 <http://dx.doi.org/10.1016/j.jff.2016.06.034>
- 553