Antioxidant activity of pasteurized and sterilized commercial red orange juices

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Blood orange juice is a typical Italian product whose red color is primarily associated with anthocyanin pigments. Two orange-based products are present on the market: pasteurized pure juice with 40 days of shelf life, and sterilized beverage containing minimum 12% of concentrated fruit juice. The aim of the present paper is to verify the relationships between the antioxidant properties and the anthocyanins content in a sampling of pasteurized and sterilized commercial red orange juices. The anthocyanins composition was determined by HPLC-MS/MS, while the antioxidant activity was evaluated by the Briggs-Rauscher reaction, selected in order to acquire information at acid pH values, by three radical scavenging assays (DMPD, 2-2′-azinobis-(3-ethylenbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), DPPH), and by FRAP assay to monitor the ferric reducing power. Results showed that antioxidant activity, particularly when measured by ABTS method, is positively related to the content of anthocyanins and that the reduction of anthocyanins content, typical of commercial long-shelf life juices, leads to a remarkable loss of antioxidant power.

Keywords: Anthocyanin / Antioxidant / Blood orange juice / Pasteurization / Sterilization

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1 Introduction

Blood orange juice is a typical Italian product, but fresh-squeezed orange juice is also currently marketed throughout the west coast of the United States. Its red color is primarily associated with anthocyanins pigments [1]. The anthocyanins are part of the plant constituents collectively known as flavonoids, which are of great nutritional interest because of their health properties and of noticeable daily dietary intake. The anthocyanins are included in the list of natural compounds known to work as powerful antioxidants.

Current literature data tendency is that anthocyanins are poorly absorbed, based on the detection of nil or very low (less than 1%) amount of anthocyanins and/or their metabolites in plasma and/or urine following the administration of anthocyanin-rich foods. However, no information has been provided concerning the metabolic fate of the rest 99% of the administered anthocyanins. Thus we believe that the absorption and the metabolic fate of anthocyanins is far to be completely elucidated. Among other factors (food matrix, nature of the sugar conjugate, and the phenolic aglycon) the understanding of the metabolic fate in the upper intestine is complicated by the poor stability of anthocyanins at intestinal pH (6 – 6.5) leading to their degradation, also depending on microbial metabolism. However, very recent *in vivo* studies demonstrated that the stomach could be a site where an important amount of dietary anthocyanins are promptly absorbed [2, 3]. In any case, a huge increase of plasma antioxidant activity is detected after ingestion of anthocyanin-rich foods [4, 5].

Several researches on *in vitro* system, animal models, and human trials demonstrated that anthocyanins present in dietary plants are able to exert protection toward tissue

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injury mediated by reactive oxygen species (ROS) [6, 7]. Besides the antioxidant action, anthocyanins and particularly cyanidins, have several biochemical and pharmacological effects, including antimutagenic, antiinflammatory, gastroprotective action, LDL protection to lipid peroxidation, and other properties extensively reviewed [6].

Pigmented oranges, named *Moro*, *Sanguinello*, and *Tarocco*, typically growing in Sicily (Italy) as well as in Florida (USA) [6] are one of the most important dietary sources of some anthocyanins, such as cyanidin-3-*O*-β-glucopyranoside (CyG), cyanidin 3-(6″-malonylglucoside), delphinidin-3-glucoside, and delphinidin-3-(malonylglucoside). Red oranges are generally perceived by Italian consumers as a healthy food, due to the presence of anthocyanins and to the higher content of ascorbic acid (about 40% more than the yellow oranges). This perception was endorsed also by frequent campaigns promoting the putative protective and anticancer properties of blood oranges. As a consequence of these marketing strategies the number of red orange juices and of soft drinks containing red orange juice as an ingredient is increasing (http://www.federalimentare.it/home.html).

Two kinds of red orange juice are commonly available on the Italian market: a short-shelf life (40 days) pasteurized and refrigerated juice (REF), containing 100% of red orange juice, and a long-shelf life (1 year) sterilized juice (LC), containing a minimal of 12% of concentrated red orange juice.

In REF juices production [8], the freshly extracted juice is immediately frozen and stored, until reprocessed. Reprocessing involves thawing, "mild" pasteurization (less than 80°C, as suggested by Marchese [9] to minimize the degradation of anthocyanins) and packaging under aseptic conditions. Distribution follows the refrigerated product chain with the above-mentioned mean shelf life of 40 days.

Blood orange anthocyanins are not very stable: during thermal treatment and storage they can degrade and form colorless or undesirable brown-colored compounds; the juice loses its bright red color and gains a brown color [10]. For this reason, sterilized commercial juices (LC) usually contain the allure red colorant.

Thermal degradation of blood orange juice anthocyanins has been studied by several authors and it can be concluded that it depends on the time/temperature of the thermal treatment and on the subsequent storage conditions: degradation is faster with increasing storage temperature [8, 11, 12].

Lo Scalzo *et al.* [13] found that the antioxidant properties of thermally treated blood orange juice was variable depending on both the kind of thermal treatment and antioxidant assay. Indeed, the inhibition of enzymatically mediated linoleic acid peroxidation was increased by thermal treatments, while the scavenging effect toward OH, generated by Fenton reaction, and (1,1-diphenyl-2-picryl-hydrazyl) (DPPH), decreased.

In any case, in contrast with the pasteurization treatment, no data are available on the effect of the sterilization treatment on the stability of anthocyanins and on the antioxidant properties of pigmented orange juice.

The aims of the present paper were to verify the relationships between the antioxidant properties and the anthocyanins content. The anthocyanins composition was determined by HPLC-MS/MS, while the antioxidant activity was evaluated by the Briggs–Rauscher (BR) reaction, selected in order to acquire information at acid pH values, by three radical scavenging assays (*N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), 2-2′-azinobis-(3-ethylenbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), DPPH), and by FRAP assay to monitor the ferric reducing power.

2 Materials and methods

2.1 Chemicals

All solvents and reagents were from Fluka AG (Switzerland).

L-Ascorbic acid, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carbosxilic acid (Trolox), and ABTS, and R-tocopherol were purchased from Aldrich (Germany), 2,6-di ter-buthyl-*p*-cresol (BHT) was from Sigma (Sigma Chemical, St. Louis, MO, USA) and from Aldrich.

DMPD and gallic acid were from Fluka.

ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride, was from Wako (Germany). All solvents (HPLC grade) were from Carlo Erba (Italy).

Malonic acid, manganese(II) sulfate monohydrate, $NaIO_3$, $HClO_4$, and H_2O_2 were purchased from Merck (Germany). All stock solutions were prepared from doubly distilled, deionized H_2O.

2.2 Sampling

Samples of all the red orange juice brands available on the Italian market were randomly purchased during 2004 in supermarkets and drugstores. Seven samples, made exclusively by pure red oranges juice, were short-shelf life (40 days) pasteurized and refrigerated juices (REF) whereas five samples, containing various amounts of con-

centrated juice ranging from 25 to 35%, were long-shelf life (1 year) sterilized juice (LC).

According to the manufacturers REF contained neither preservatives nor colorant, whereas all the samples of LC contained colorant (E129 Allure Red or E122 Azorubine). All the refrigerated samples were tested immediately after purchasing.

2.3 Analytical procedures

Color assay, pH, and vitamin C were determined on samples immediately after the pack opening. Samples were centrifuged at 4000 rpm for 10 min and the supernatant was used for anthocyanins determination and antioxidant activity assays.

2.4 Determination of vitamin C

Vitamin C was determined by titration with DIF (2,6-dichlorophenolindophenol sodium salt hydrate) using the official method described by the AOAC [14].

2.5 Determination of color density, polymeric color, and percent polymeric color

Indices for density, polymeric color, and percent polymeric color in the samples were determined using the method of Giusti and Wrolstad [15]. The juice samples were diluted five-fold with a 25 mM solution of potassium chloride at pH 1 until the absorbance at 520 nm was < 1. Diluted samples (2.8 mL) were transferred into two cuvettes; 0.2 mL of sodium metabisulfite solution (0.90 M) was added into one cuvette, and 0.2 mL of distilled water was added to the other.

After 15 min, the absorbance of both samples was measured at 420, 520, and 700 nm and compared with a blank containing distilled water. Color density of the control sample (distilled water) was calculated as

$$\text{Color density} = [(A420\ \text{nm} - A700\ \text{nm}) + (A520\ \text{nm} - A700\ \text{nm})] \times 5$$

Polymeric color of the bisulfite-bleached sample was calculated as

$$\text{Polymeric color} = [(A420\ \text{nm} - A700\ \text{nm}) + (A520\ \text{nm} - A700\ \text{nm})] \times 5$$

Percent polymeric color was calculated using the formula

$$\%\ \text{polymeric color} = (\text{polymeric color}/\text{color density}) \times 100$$

2.6 Determination of anthocyanins

The HPLC method for detection of anthocyanins was as described by Gennaro *et al*. [16] with some modifications. A Supelcosil LC-18 column (250 × 4.6 mm ID, 5 μm) with a Spherisorb Supelguard LC-18 (Supelco, USA) was used. Chromatography was performed at room temperature, at a flow rate of 0.8 mL/min using the following solvent system: solvent A (H_2O TFA 0.5%), solvent B (methanol). Column was equilibrated in 20%B and anthocyanins elution was achieved by the following linear gradient: 12 min 60% of B, 3 min 70% of B, 15 min of isocratic elution (30%A, 70%B). The elution of all anthocyanin compounds was monitored at 520 nm. Quantitation of single compounds was achieved by a calibration curve obtained using pure kuromanine (cyanidin-3-glucoside) as a standard (Extrasynthese, France).

The chromatographic instrumentation consisted of two LC-10ADvp series pumps (Shimadzu, Kyoto, Japan), an SCL-10Avp system controller, and Shimadzu SPDM10Avp DAD detector. Data were processed by CLASSVP 5.3 Shimadzu software.

2.7 LC/MS/MS experiment

MS/MS data were obtained by an API 3000 triple-quadrupole mass spectrometer (Applied Biosystem, Toronto, Canada) equipped with a TurboIonspray source.

The operating parameters were as follows: capillary voltage +5000 V, focus potential +320 V, declustering potential (DP) +45 V, collision energy (CE) +40 V, and temperature 400°C.

Acquisition was performed by multiple reaction monitoring (MRM) in positive ion mode.

MS/MS product ions were produced by collision-activated dissociation (CAD) of a selected precursor ion in the collision cell of the mass spectrometer, and then analyzed using the third quadrupole. LC analysis was carried out with two micro pump series 200 Perkin Elmer (Canada). The column and the chromatographic conditions were the same as described above, but 200 μL/min into the ion source was splitted.

2.8 Antioxidant activity assay based on the BR reaction

Antioxidant activity of red orange juices was measured using the chemical *in vitro* method reported by Cervellati *et al.* [17], which is based on the inhibitory effects by ROS scavengers on the oscillations of the BR reaction.

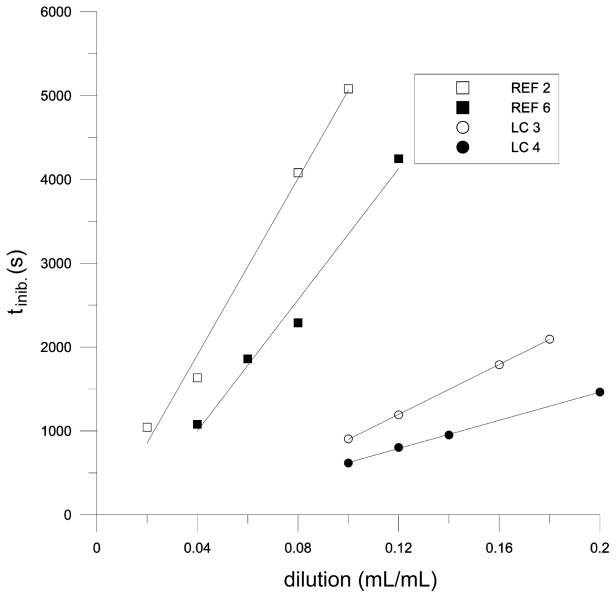

Figure 1. Typical behaviour of $t_{inhib.}$ *versus* dilution (mL of suitable diluted juice in the mL added to the BR mixture) for two REF and two LC red orange juices.

The BR system [18] consists of hydrogen peroxide, acidic iodate, malonic acid, Mn(II) as catalyst, and works at pH ≈ 2. The reaction method is based on the generation of free radicals in the reaction mixture. The generated hydroperoxyl radicals ($HOO^{\bullet}$) are among the main intermediates of the BR system. The mechanism of the action of antioxidants against $HOO^{\bullet}$ radicals in the BR system has been described in detail elsewhere [17–19]. In brief, when antioxidant scavengers of free radicals are added to an active oscillating BR mixture there is an immediate quenching of the oscillations, an inhibition time that linearly depends on the concentration of the antioxidant added, and a subsequent regeneration of the oscillations. Typical graphs t_{inhib} *versus* dilution for some samples are reported in Fig. 1. Relative antioxidant activities with respect to a substance chosen as a standard are determined on the basis of the inhibition times. Diluted (1:5) samples were further diluted (0.02–0.2:1) and 1.0 mL of these diluted solutions was added to 30 mL of an active BR mixture after the third oscillation. More details about the experimental procedure and relative antioxidant activity calculation have been reported in [20].

2.9 Assay of antioxidant activity based on colored radical cations

The water soluble antioxidant activity of the red orange juice was measured using the DMPD method as described by Fogliano *et al.* [21], while the antioxidant activity of the methanol extracts was measured by the ABTS method performed as previously described [22] and the DPPH method was carried out as described by Zhu-Qiu [23]. To measure antioxidant activity of each sample, aqueous and methanol dilutions of red orange juices were obtained. Aqueous dilution was used for the DMPD assays while methanol dilution for the ABTS and DPPH assays. The dilutions were performed as follows: 1 mL of each sample was added to 5 mL of distilled water or methanol and then put in refrigerated centrifuge at 4000 rpm for 5 min. The procedure was performed twice. Precipitates were discarded and the supernatants were put in ice bath and immediately used for antioxidant assays.

2.10 Assay of ferric reducing activity

The ability of red orange juice to reduce ferric ion was measured by the ferric reducing antioxidant power (FRAP) method, carried out as described by Benzie and Strain [24].

3 Results and discussion

A sampling of commercial red orange juice was carried out during winter 2004. All samples collected had more than 20 days (for REF samples) and of 8 months (for LC samples) of residual shelf life, respectively. Data of the collected sample are shown in Table 1. The pH is slightly lower in LC than in REF, ranging between 3.12 and 3.34 for REF and between 2.66 and 3.20 for LC.

Table 1. % juice, pH, vitamin C, color, % polymeric color of the red orange juice sample. Pasteurized refrigerated juices (REF), sterilized juice (LC)

Sample	% juice	pH	Vitamin C, mg/100 mL	Color density index	% polymeric color
REF 1	100	3.30	81.1	5.97	13.9
REF 2	100	3.27	75.5	6.47	14.8
REF 3	100	3.34	62.6	3.97	17.9
REF 4	100	3.13	46.8	3.66	19.4
REF 5	100	3.12	70.5	5.39	14.5
REF 6	100	3.27	83.7	5.62	17.3
REF 7	100	3.17	38.9	6.56	33.4
LC 1	35	2.92	54.7	2.06	89.6
LC 2	35	2.87	60.0	2.77	99.8
LC 3	25	2.66	89.0	3.89	93.1
LC 4	30	3.03	75.8	1.40	84.6
LC 5	26	3.20	75.5	2.72	91.5

No differences were detected for vitamin C, which was between 38.9 and 89.0 mg *per* 100 mL of juice. The measure of color density and of polymeric color gave an immediate distinction between the two groups of samples. In REF samples the color density was higher and the percentage of polymeric color was considerably lower than in LC samples. This clearly indicates that the color of steri-

Table 2. Concentration of anthocyanins in fruit juices

Sample	Total anthocyanins, mg/L	Cyanidin-3-glucoside (CyG), mg/L	Malonyl-3-glucoside, mg/L
REF 1	75.5	29.7	25.27
REF 2	97.7	35.5	35.55
REF 3	44.7	17.0	15.61
REF 4	43.0	14.6	17.58
REF 5	72.0	24.8	28.23
REF 6	62.2	22.0	24.62
REF 7	67.2	23.5	25.62
LC 1	0.9	0.7	1.14
LC 2	0.4	0.4	0.08
LC 3	2.0	1.6	0.40
LC 4	3.3	1.1	0.34
LC 5	5.4	1.2	0.63

lized red orange juice is mainly due to the presence of chemical colorant added.

The pattern of anthocyanins in REF and LC samples was investigated by HPLC. The pattern of anthocyanins in REF and LC samples was investigated by HPLC-MS/MS. In the REF samples (panel A) the major compounds are CyG and cyanidin 3-(6″-malonylglucoside), in agreement with literature data [25, 26]. In the LC samples almost no anthocyanins were detected, thus confirming that the commercial sterilization process leads to the degradation of anthocyanins. The concentrations of CyG and cyanidin 3-(6″-malonylglucoside) together with the total amount of anthocyanins detected in REF and LC samples are shown in Table 2. Besides the huge differences between the two typologies a marked variability among the REF samples can also be observed due to the different quality of the raw material. It must be evidenced that anthocyanins concentration in REF juices is comparable to that reported for fresh red orange juice [11, 27], thus confirming that the pasteurization treatment and storage condition applied to this category of commercial samples do not damage anthocyanins. On the other hand, the content of anthocyanins in the LC samples is almost negligible. This is partly due to the lower percentage of fruit juice present in the beverage (between 25 and 35% according to the indication of the manufacturers), and also to a severe degradation of the anthocyanins. Literature data show that the loss of anthocyanins in red fruit juices is negligible when heat processing is carried out for less than 12 min at 100°C [28 – 30]. However, the degradation of red orange anthocyanins is more pronounced. In fact, in a test performed to determine the degradation kinetics of anthocyanins in blood orange juice during heating and storage at various temperatures, Kirca *et al.* [31] demonstrated that the losses of anthocyanins were 14.4, 21.5, and 60.9% at the end of 120 min heating at 70, 80, and 90°C, respectively, suggesting that blood orange juice anthocyanins are susceptible to the above-mentioned time/temperature ranges of thermal treatment. They also confirmed that anthocyanin

Table 3. ESI-MS-MS fragmentation data in positive mode of the anthocyanins present in the red orange juice sample (REF 1)

Compounds	r.t.	M_r	MS/MS ions
Pelargonidin diglucoside	10.15	595	433.271
Cyanidin diglucoside	11.14	611	449.287
Cyanidin glucoside	11.95	449	287
Pelargonidin glucoside	12.69	433	271
Delphinidin malonyl galactoside	13.25	551	389
Delphinidin malonyl glucoside	13.90	551	389
Cyanidin malonyl glucoside	14.05	535	373.287
Pelargonidin rhamnosil glucoside	14.10	579	417.271
Cyanidin arabinoside	14.26	419	287
Pelargonidin malonyl glucoside	14.70	519	357.271
Cyanidin rhamnosil glucoside	15.30	595	433.287

levels decreased very rapidly for samples stored at 37 and 20°C whereas the samples stored at 5°C showed a remarkably slower degradation. Choi *et al.* [32], after pasteurizing at 90°C for 90 s, observed a 25% decrease of the level of total anthocyanins after 7 wk of storage at 4.5°C. Therefore, it can be concluded that the combination of severe thermal treatment and storage at room temperature is responsible for the absence of anthocyanins in LC samples.

The HPLC-MS/MS also allows to perform a tentative identification of the other anthocyanins compounds present in the chromatograms of REF samples. In Table 3 the ESI-MS/MS results are summarized showing the identified compounds, the retention time, *m/z* of each precursor ion [M], and the monitored ions. Data were obtained on the sample REF1, but only minor differences were detectable among the other REF samples. Eleven anthocyanins were identified, together with the two major compounds (cyanidin-3-glucoside and cyanidin-3-malonylglucoside), others glycosides of cyanidin were also present such as cyanidin diglucoside, cyanidin rhamnosil glucoside. Minor amounts of cyanidin arabinoside, pelargonidin rhamnosil glucoside, delphinidin malonyl galactoside, and pelargonidin glucoside were also detected. All of the masses found are in

Table 4. Antioxidant activity measured by BR, DMPD, FRAP, ABTS, DPPH

Sample	BR DHBAE, mg/L	DMPD Ac. Asc., mM	FRAP Fe (II), mM	ABTS Trolox, mM	DPPH Trolox, mM
REF 1	920	409.2	21.5	3.13	2.49
REF 2	870	432.5	21.1	3.53	3.08
REF 3	750	323.7	17.6	2.54	1.6
REF 4	590	395.1	15.0	2.12	1.17
REF 5	585	477.2	20.8	3.18	2.51
REF 6	530	400.2	18.9	2.98	3.01
REF 7	560	404.1	20.0	2.28	1.26
LC 1	370	155.0	4.3	0.60	0.25
LC 2	210	214.5	3.3	0.54	0.21
LC 3	195	213.5	8.7	0.70	1.38
LC 4	175	68.6	5.3	0.41	0.34
LC 5	150	196.1	4.4	0.35	0.27

Table 5. Pearson correlations between the 12 juice samples

	ABTS	DPPH	DMPD	FRAP	BR	Total anthocyanins	CyG	Vitamin C	pH	Polymeric color
ABTS		0.920	0.927	0.971[a)]	0.913	0.971[a)]	0.972[a)]	0.044	0.690	−0.963[a)]
DPPH			0.821	0.881	0.770	0.878	0.885	0.382	0.518	−0.826
DMPD				0.924	0.805	0.913	0.900	−0.108	0.571	−0.899
FRAP					0.881	0.959[a)]	0.960[a)]	−0.009	0.659	−0.956[a)]
BR						0.887	0.910	−0.054	0.691	−0.897
Total anthocyanins							0.997[b)]	0.002	0.703	−0.921
CyG								0.030	0.699	−0.917
Vitamin C									−0.036	0.083
pH										−0.757

a) Correlation is significant at the 0.05 level (two-tailed).
b) Correlation is significant at the 0.01 level (two-tailed).

agreement with the data shown in literature for both classes of compounds, the monoglycosides and diglycosides [33].

Anthocyanins are powerful antioxidants, therefore it can be expected that their strong reduction in the LC resulted in a marked reduction of antioxidant activity. As it is well-known that the numerous methods available to assess the antioxidant activity have different sensitivity for the various compounds present in the orange juice, diverse assays should be adopted to evaluate this parameter. Overall, *in vitro* chemical antioxidant assays can only partially mimic physiological conditions. The BR reaction method was adopted because it works at a pH (≈2) very close to that of the stomach that has been recently demonstrated to be a site where an important amount of dietary anthocyanins are promptly absorbed [2, 3]. The BR relative antioxidant activity as equivalents of 2,6-DHBA (2,6-dihydoxy benzoic acid) in mg/L (DHBAE) for the samples of red orange juice is reported in Table 4; it is evident that the values are very different for the two groups of juices with the value of REF samples significantly higher than that of LC samples.

Results of the antioxidant activity obtained with the other methods are summarized in Table 4.

All assays gave clearly different values for the two groups of juice, REF samples having a higher antioxidant power than LC ones. For DMPD scavenging effect the mean value, expressed in mM of ascorbic acid, was 406.0 for REF samples and 169.5 for LC samples; for ABTS and DPPH assays the mean values, expressed in mM of Trolox were, respectively, 2.82 for REF samples and 0.52 for LC samples, and 2.16 for the REF samples and 0.49 for the LC samples; for FRAP assay the mean value, expressed in mM of Fe(II), was 19.27 for REF samples and 5.2 for LC samples.

Pearson's correlation coefficients among polymeric color, total anthocyanins, CyG, vitamin C, pH, and all antioxidant assays were calculated and are reported in Table 5. The total concentration of anthocyanin results is positively correlated with ABTS values and, obviously, to the CyG content (coefficients 0.971 and 0.972, respectively). ABTS is also correlated with FRAP value (coefficient 0.971) and negatively related to polymeric color (coefficient −0.963). All the other antioxidant assay value do not reach the $p = 0.95$ in the correlation with total anthocyanin or with CyG.

Therefore, although all methods detected the huge difference between the REF and LC samples, it can be concluded that ABTS, being more sensitive to the anthocyanins, can be preferentially used to rapidly assess the influence of process and storage on the concentration of these compounds. Moreover, the major role exerted by anthocyanins on the antioxidant activity also at acidic pH suggests that these compounds can be of particular importance to prevent the oxidation reaction in the stomach where a tremendous amount of oxygen and nitrogen radicals are produced during food digestion.

In conclusion, this study has shown a severe loss of anthocyanins content in LC red orange juice beverage presumably due to the condition of thermal treatments and storage. From the consumers point of view our findings are of a certain importance. Indeed, as mentioned above, consumers perceive red oranges as a healthy food, and the antioxidant properties of red oranges certainly play a fundamental role in their choice of purchase. However, the healthy properties related to the antioxidant properties exerted by anthocyanins can be attributed only to the short-shelf life product containing 100% pure juice.

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