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Effect of thermal treatments on antioxidant and antiradical activity of blood orange juice

R. Lo Scalzo[a,*], T. Iannoccari[a], C. Summa[a], R. Morelli[b], P. Rapisarda[c]

[a] *Istituto per la Valorizzazione Tecnologica dei Prodotti Agricoli, via G. Venezian, 26-20133 Milan, Italy*
[b] *Istituto di Scienze e Tecnologie Molecolari CNR, via Golgi, 19-20133 Milan, Italy*
[c] *Istituto Sperimentale per l'Agrumicoltura, Corso Savoia 190-95024 Acireale, Italy*

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Abstract

Blood orange juice is noted for its antioxidant properties, due to its rich phenolic profile. Orange segments were subjected to brief thermal treatments before transformation into juice, and changes in antioxidant and chemical composition were evaluated. Thermal treatment generally induced an increase in the main phenolic substances of orange juice, such as anthocyanins and total cinnamates, while ascorbic acid underwent a decrease. Antioxidant properties, evaluated in a lipoxygenase–linoleic acid system, were higher in thermally-treated samples, while free radical scavenging activity, evaluated by ESR spin trapping of hydroxyl radical and DPPH· quenching, were enhanced in untreated juices. A possible relationship between the changes in composition and the changes in antioxidant and antiradical properties is suggested.

Keywords: Blood orange juice; Thermal treatments; Antioxidants; Free radical scavengers

1. Introduction

Blood oranges, 'Tarocco', 'Sanguinello' and 'Moro', are the main cultivated varieties of *Citrus sinensis* (L.) Osbeck in Sicily. These cultivars are characterized by their unique flesh and rind colour due to phenolic pigments belonging to the anthocyanin class (Maccarone, Maccarrone, Perrini, & Rapisarda, 1983; Maccarone, Maccarrone, & Rapisarda, 1985). Blood orange fresh tissue and juice contain a predominance of cyanidin-3-glucoside and cyanidin-3-(6″-malonyl)-glucoside (Maccarone, Rapisarda, Fanella, Arena, & Mondello, 1998). These water-soluble pigments are known as contributors to antioxidant activity (Arena, Fallico, & Maccarone, 2001; Bonina et al., 1998; Rapisarda, Tomaino, Lo Cascio, Bonina, De Pasquale, & Saija, 1999) and antiradical activity (Miller, Diplock, & Rice-Evans, 1995; Wang, Cao, & Prior, 1997). Also, other blood orange components (ascorbic acid, flavonoids, and hydroxycinnamic acids) have high antioxidant capacities (Arena et al., 2001; Gardner, White, McPhail, & Duthie, 2000; Rapisarda, Bellomo, & Intrigliolo, 2001).

These compounds constitute useful markers to allow recognition and evaluation of nutritional quality in fresh and processed products. Furthermore, there is clear epidemiological evidence of a relationship between fruit and vegetable consumption and health benefits (Ames, Shigena, & Hagen, 1993). In such benefits, the antioxidant and radical scavenging properties of some food components are surely involved.

Fruits and vegetables are often subjected to thermal treatments in order to preserve their quality, especially by minimizing undesirable enzyme activities, often using peroxidase activity as a marker (Moulding, Grant, McLellan, & Robinson, 1987; Pizzocaro, Ricci, & Zanetti, 1988). These treatments are also frequently useful for the better extraction of antioxidant substances, as already found on other vegetables (Gazzani, Papetti, Masolini, & Daglia, 1998; Pizzocaro, Gasparoli, & Ambrogi, 1995; Pizzocaro, Russo Volpe, Morelli, & Oliva, 1997).

The present study was aimed at evaluating the effects of some thermal treatments on antioxidant and antiradical

* Corresponding author. Tel.: +39-02-239-557211/210; fax: +39-02-236-5377.
E-mail address: testoni.ivtpa@interbusiness.it (R. Lo Scalzo).

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activity of 'Moro' orange juice obtained from orange segments processed by different methods.

In addition, juices were analysed for their content of some antioxidant products, in order to check the relationship between the changes in antioxidant and antiradical activities induced by thermal treatments and the changes in chemical composition.

2. Materials and methods

2.1. Plant materials

The study was carried out on oranges belonging to the 'Moro' cultivar, harvested in March 2001 in the experimental field of the Istituto Sperimentale per l'Agrumicoltura (Acireale, Italy).

Once in the laboratory, oranges (about 16 kg) were divided into four homogeneous groups. Successively, the fruits were manually peeled and reduced to segments. The segments from each aliquot were separately processed according to four different methods:

1. the segments were squeezed and the juice produced was not thermally treated (NT);
2. the segments were subjected to blanching at 80 °C for 6 min before squeezing (Blanch);
3. the segments were squeezed and the juice successively pasteurized at 80 °C for 1 min (Past);
4. the segments were subjected to blanching, then squeezed and the juice pasteurized (Blanch-Past).

In all of the tests, segments of orange were cloth-pressed at 200 kg cm^{-2} in a pilot machine of 2 kg capacity, to obtain two replicate samples of juice for each treatment.

2.2. Quality attributes

Total soluble solids (TSS), pH and total acidity (TA) were measured in each of three replicates, using the juices obtained according to the four different methods cited above.

Total soluble solids, pH and TA were determined by standard methods (MAF, 1989).

Juice colour was measured using a Minolta Chromameter CR-310 (Minolta Camera Co. Ltd., Osaka, Japan) to determine CIELAB parameter a^* (redness), b^* (yellowness), L^* and a^*/b^* ratio.

2.3. Determination of anthocyanins

Total anthocyanin content in fruit juices was determined by the pH differential method (Rapisarda, Fallico, Izzo, & Maccarone, 1994). Results were expressed as mg/l of cyanidin-3-glucoside.

HPLC analysis was performed with a Jasco Bip-I solvent pump equipped with a Jasco GP-A30 gradient programmator, a Jasco UVIDEC-100-V detector and a C-R6A Chromatopac Shimadzu integrator. An Inertsil PH column 250 × 4.6 mm i.d. and 5 μm particle diameter was used and the mobile phase systems were the following: solvent A, MeOH–10% acetic acid (20:80, v/v), and solvent B, 10% acetic acid at a flow rate of 0.6 ml/min. A linear gradient from 1 to 30% of A was carried out in 35 min. The column temperature was maintained at 35 °C and analysis monitored at 525 nm.

The peak assignments were based on the presence of two major peaks [cyanidin-3-glucoside and cyanidin-3-(6″malonyl)-glucoside] in the chromatograms, after comparison with literature data.

2.4. Determination of hydroxycinnamate derivative

Total (glycosylated and free) hydroxycinnamic acids were extracted from orange juice and spectrophotometrically analysed according to Dao and Friedman (1992) with some modifications. Briefly, 25 ml of absolute ethanol were added to an aliquot of juice (5 g) and homogenized with an Ultra-Turrax T25 at 20 500 rpm for 20 s. This mixture was stored at 2 °C for 30 min and then centrifuged with a Beckman J2-21, rotor J2-20 at 8000 rpm for 10 min.

The supernatant was put in a 150-ml separator funnel and 50 ml of *n*-hexane were added. The mixture was shaken and was then left to separate into two phases. The hydroalcoholic solution volume was adjusted to 50 ml with absolute ethanol and diluted 1:4 with the same solvent. A Unicam UV/vis Spectrophotometer and 1 cm quartz cells were used throughout. Absorbance was measured at 330 nm. The results were expressed as mg chlorogenic acid equivalents/100 g juice using a molar extinction coefficient of 18,130 cm^{-1} M^{-1}. Each assay was performed in quadruplicate.

Free caffeic, chlorogenic and ferulic acids of blood orange juice were separated and determined by HPLC using the same equipment as described above. A solution of water–methanol–acetic acid (60:36:4 v/v/v) was used as mobile phase at a flow rate of 0.9 ml/min. The column temperature was maintained at 35 °C, samples of 20 μl were injected and the chromatograms were recorded at 330 nm. The peak assignments were based on the retention time of the standards. Calibration curves of peak areas versus known concentrations of standards were plotted. Concentrations of acids, expressed as mg/l, were calculated from the experimental peak areas by analytical interpolation with the calibration curves. Each assay was performed in quadruplicate.

2.5. Determination of ascorbic acid

Ascorbic acid was determined by HPLC using the same equipment as described above. The analytical column was a 250 × 6 mm i.d., Intersil ODS-3, maintained at 20 °C. The mobile phase consisted of orthophosphoric acid 0.02 M at a flow rate of 0.7 ml/min and samples of 5 μl were injected and monitored at 254 nm. An aliquot of freshly prepared juice (5 ml) was diluted to 25 ml using 6% metaphosphoric acid, filtered through a 0.45-μm Whatman Puradisc and HPLC injected.

The concentration of ascorbic acid was calculated from the experimental peak area by analytical interpolation in a standard calibration curve, and was expressed as mg/100 ml of orange juice. Each assay was performed in quadruplicate.

2.6. Peroxidase activity

Soluble and ionically bound peroxidase activities (PODs,i) were determined by the spectrophotometric methods of Gkinise and Fennema (1978) and Pütter (1974), on a Jasco Uvidec-320 spectrophotometer with 1 cm quartz cells at 436 nm and at 25 °C.

For soluble peroxidase (PODs), 10 g of orange juice were diluted with 20 ml of phosphate buffer 0.1 M (pH 7.0), while, for ionically bound (PODi), 10 g of orange juice were added to 10 ml of 1 N NaCl.

The POD assay mixture contained 2.9 ml of 0.1 M phosphate buffer (pH 7.0), 0.005 ml of 20.1 mM guaiacol as donors, 0.03 ml of 12.3 mM H_2O_2 as substrate and 0.2 ml of orange juice extract. The enzyme activity was determined by measuring the slope of the reaction line and one POD unit was defined as 0.001 ΔDO_{436} min^{-1} (DO = optical density).

Inhibition activity was expressed as % of residual activity, referred to a sample not subjected to any thermal treatment being equal to 100%.

2.7. Lipoxygenase activity

The lipoxygenase activity was determined using the method of Grossman and Zakut (1979). The measure was carried out using, as enzymatic extract, the orange juice 'NT' and, as substrate, linoleic acid (Merck, Darmstadt).

2.8. Lipoxygenase–linoleic acid method for antioxidant activity (AA)

In this method, the enzymatic oxidation of linoleic acid was obtained by an external addition of lipoxygenase by modifying the method of Grossman and Zakut (1979; Pizzocaro et al., 1995). The oxidation of linoleic acid was analysed in the absence (blank test) and in the presence (sample test) of the four samples of orange juice by recording the linear increase in absorbance at 234 nm.

In the sample test, the lipoxygenase inhibition of orange juice was measured in a solution made up of 2.65 ml of 0.1 M phosphate buffer (pH 7.0), 0.3 ml of 2.28 mM linoleic acid–water emulsion, 0.05 ml of orange juice and 0.025 ml of the lipoxygenase solution made by dissolving 15 mg lipoxygenase standard extract of soybean (Sigma, St. Louis, USA) in 25 ml of 0.1 M phosphate buffer (pH 7.0). This solution was slowly shaken and stored at 2 °C until used.

In the blank test orange juice was substituted with 0.05 ml of 0.1 M phosphate buffer (pH 7.0).

AA was expressed as protection percentage from the enzymatic degradation of linoleic acid referred to the blank, considered as 100% degradation. The inhibition percentage in the presence of orange juice was considered as the antioxidant activity. Each assay was performed in quadruplicate.

2.9. Evaluation of radical scavenger activity by electron spin resonance (ESR) spectrometry

Active oxygen species, such as the hydroxyl radical (OH•), are formed through a one-electron reduction of hydrogen peroxide (H_2O_2). The OH• is generated by a process known as redox cycling or Fenton reaction, and is catalysed by transition metal such as Fe^{2+}:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$

The hydroxyl radical in the reaction mixture was trapped with 5,5-dimethylpyrrolidine-*N*-oxide (DMPO), and the resultant adduct DMPO–OH• was detected by an ESR spectrometer Varian E-line Century series, a very useful method to detect the presence of hydroxyl and others free radicals (Buettner, 1982; Rimbah et al., 1999). The adduct, DMPO–OH•, gave four vertical signals in a plot magnetic field vs arbitrary resonance units (Fig. 1a).

The ESR spectrometer was set at the following conditions: frequency 9.26 GHz, power 10 mW, field set 3390 Gauss, scan time 2 min, time constant 0.5 s, gain 16,000, modulation: 1 Gauss p–p. ESR spectra were recorded 2 min after mixing 0.2 ml of orange juice diluted 1:5 with 0.1 M phosphate buffer (pH 7.4), 0.2 ml of 10 mM H_2O_2, 0.2 ml of 10 mM Fe^{2+} and 0.2 ml of 50 mM DMPO. For OH• measurement, ESR spectra of a mixture without and with the scavenging sample were recorded, where the signal height is strictly related to the OH concentration.

The scavenger activity of the test sample for OH• was expressed by the following formula:

$$I = 100 - [(h_x/h_o)]^*100$$

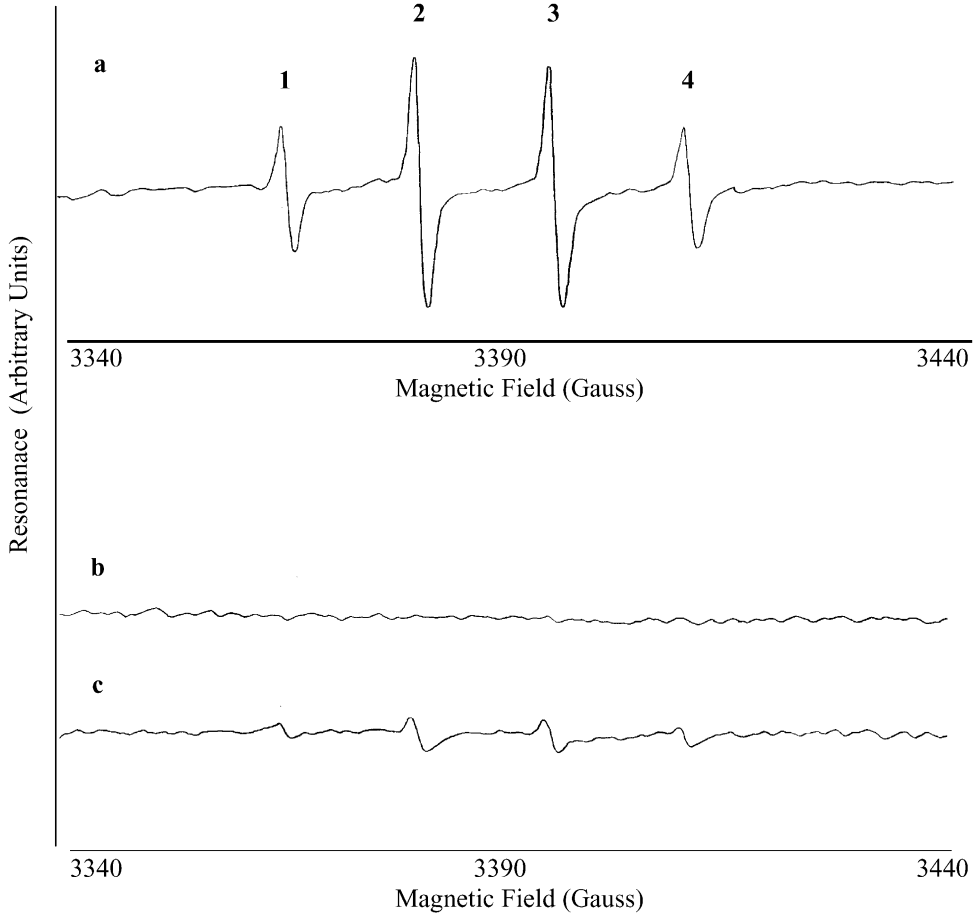

Fig. 1. ESR spectrum of a DMPO–$OH^{\cdot}$ adduct in a blank sample without scavenging material (a), with an undiluted blood orange juice (b) and with a 5-fold diluted blood orange juice (c). Spectra are registered on the same scale (Arbitrary Resonance Units).

where I is scavenger activity, h_o and h_x are the relative heights of the signal nr. 2 (mm) (Fig. 1a) in a reaction mixture without and with the scavenger solution, respectively. Each assay was performed in quadruplicate.

2.10. Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical

Scavenging activity of blood orange juices was measured by DPPH• radical quenching (Brand-Williams, Cuvelier, & Berset, 1995; Rapisarda et al., 1999).

Twenty millilitres of methanol were added to an aliquot of juice (10 g) and homogenized with the Ultra-Turrax T25 at 20,500 rpm for 25 s. Subsequently, this mixture was centrifuged with a Beckman J2-21, rotor J2-20 at 20,000 rpm at 4 °C for 25 min. The supernatant was diluted 1:25 with methanol. The extract (1.0 ml) was dissolved in 1.0 ml methanol and added to 0.5 ml methanolic solution containing DPPH• 0.5 mM. The control sample was prepared using 2.0 ml methanol and 0.5 ml of the same methanolic solution containing DPPH•. The methanolic solution containing DPPH• was kept at room temperature in the dark. The DO of the remaining DPPH• was measured in a 1 cm cuvette with a Unicam UV/vis spectrophotometer at 517 nm and at 25 °C. The reaction mixture was shaken and left to stand for 15 min at room temperature in the dark. The radical scavenging activity (S) of each extract was expressed by the following formula:

$$S = 100 - [(A_x/A_o)]^*100$$

where A_x was the optical density of DPPH• solution in presence of orange juice and A_o the optical density of DPPH• solution in the absence of the sample. Each assay was performed in quadruplicate.

3. Results and discussion

The efficiency of thermal treatments on blood orange juices was initially assayed (data not shown) with a peroxidase test according to Gkinise and Fennema (1978) and Pütter (1974), because the thermoresistance of this enzyme is well known (Moulding et al., 1987; Pizzocaro et al., 1988). In an extract of fresh orange tissue, only ionically bound peroxidase was found, whose activity was completely inhibited by thermal

treatment of segments of 6 min at 80 °C. The juice not subjected to any thermal treatment showed no peroxidase activity; however the blanching time, based on the previous data, was established as 6 min.

The main quality parameters (Table 1) showed no great changes among the juice samples, except for the low value of pH in 'NT', supported by a high value of total acidity and the highest value of a^*/b^* with a low value of L^* in 'Blanch-Past', indicating a better brilliant red colour of the juice.

The increase in anthocyanin content was generally evident in thermally-treated juices, especially for 'Past' and 'Blanch-Past' samples. Only the blanching process led to a slight decrease in the total anthocyanin concentration. In the first three tests, the ratio of concentration between cyanidin-3-glucoside and cyanidin-3-(6″-malonyl)-glucoside in the juices remained more or less constant, while the increase in cyanidin-3-glucoside and the decrease in cyanidin-3-(6″-malonyl)-glucoside in the 'Blanch-Past' sample, seem to demonstrate the hydrolysis of the malonyl group bound to glucose, due to the technological process.

The amount of hydroxycinnamates differed according to the method used. The HPLC analysis was able to find only the free hydroxycinnamic acids, while the spectrophotometric test gave the absorbance of all substances with a hydroxycinnamic fraction, especially in ester and glycoside forms.

The results of spectrophotometric analysis clearly indicated an increase in hydroxycinnamates due to the thermal treatments. It is evident that the presence of significant amounts of hydroxycinnamates in juice sac membranes (Manthey & Grohmann, 2001) leads to an increase of these products in solution owing to thermal treatments.

Single compounds, evaluated by HPLC, gave the same result only for caffeic acid and, partially, for ferulic acid, whereas only in the 'Past' sample were statistically higher values of chlorogenic acid observed.

Initially, in order to check the possible presence of the lipoxygenase in the juice, the enzyme activity was assayed on 'NT' juice according to the test planned by Grossman and Zakut (1979). The lipoxygenase activity was absent by the reported method (data not shown); hence it was concluded that the test of antioxidant activity (AA), based on linoleate degradation, had to be carried out in the presence of an external source of lipoxygenase.

The results of this test are shown in Table 2. All juice samples showed a significant antioxidant activity with respect to a blank to which only a phosphate buffer was added. The increase in absorbance of the blank test at 234 nm, due to conjugated diene formation, was inhibited by about 50% in the sample tests, but the thermally-treated juices showed significantly more activity than the 'NT' juices.

This situation implies that a higher antioxidant capacity of thermally treated juices can be ascribed to the extraction, during processing, of antioxidant compounds, such as free and bound hydroxycinnamic acids and anthocyanins.

Table 2
Test of lipoxygenase-linoleic acid (AA): average values of antioxidant activity as inhibition of diene formation at 234 nm from linoleic acid by soybean lipoxygenase in the presence of blood orange juice obtained according to different methods

Sample	ΔDO/min (234 nm)	Inhibition (%)
Blank	1.16c	0.00c
NT	0.61b	47.9b
Blanch	0.52a	55.2a
Past	0.49a	57.8a
Blanch-Past	0.56a	51.7a

Different letters for each column correspond to statistically different values ($P < 0.05$).

Table 1
Main chemical quality parameters of blood orange juice samples obtained after squeezing of segments processed according to different methods

	NT	Blanch	Past	Blanch-Past
Total soluble solids (g/100 g)	10.96b	9.87a	10.88b	9.58a
pH	3.51a	3.72b	3.57a	3.65b
Total acidity (meq/100 g)	10.6b	8.01a	10.1b	8.31a
L^*	31.64a	32.13ab	33.43b	31.33a
(a^*/b^*)	5.16b	5.24b	3.83a	7.05c
Ascorbic acid (mg/100 ml)	44.27b	41.69a	42.69a	38.62a
Total Anthocyanins (mg/l)	55.9a	50.8a	82.5b	64.2b
Cyanidin-3-glucoside (% of HPLC area)	14.3a	16.9b	17.0b	25.6c
Cyanidin-3-(6″-malonyl)-glucoside (% of HPLC area)	85.7a	83.1a	82.9b	74.37c
Total hydroxycinnamate spectrophotometric assay (mg/100 g)	30.3a	37.6b	35.8b	35.3b
Chlorogenic acid (HPLC) (mg/l)	24.2a	23.3a	30.1b	22.6a
Caffeic acid (HPLC) (mg/l)	3.7a	3.9ab	4.2b	4.5b
Ferulic acid (HPLC) (mg/l)	3.1a	3.9b	2.9a	4.0b
Total hydroxycinnamate (HPLC) (mg/l)	31.0a	31.1a	37.2b	31.1a

Average values of four replicates; different letters for each line correspond to statistically different values ($P < 0.05$).

Table 3
Antioxidant activity against free radicals of blood orange juice samples[a]

Samples	Inhibition OH• (%) (juices diluted 5-fold)	Inhibition DPPH• (%) (juices diluted 75-fold)
Blank	0.00	0.00
NT	85.0b	49.1b
Blanch	81.1a	43.1a
Past	81.5a	43.2a
Blanch-Past	83.3ab	42.9a

Different letters for each column correspond to statistically different values ($P < 0.05$).

[a] Average values of radical-scavenging activity (RSA) as inhibition of OH• formation, detected by ESR of its DMPO adduct and, as DPPH• quenching, detected spectrophotometrically at 517 nm in the presence of blood orange juice obtained according to different methods.

The radical-scavenging activity (RSA) of the blood orange juices, obtained according to four different methods, was assessed by means of two in vitro tests: inhibition of OH• formation detected by ESR and DPPH• quenching evaluated at 517 nm.

All juice samples showed a significant RSA with respect to the blank (Table 3). In both tests, the 'NT' juice showed significantly higher values of this parameter than thermally treated juices. The OH• inhibition was very high, about 80%, when juice was five-fold diluted before adding to the reaction mixture (Fig. 1c), giving the un-diluted juice a spectrum without any signal (Fig. 1b). The 'NT' juice gave a scavenging effect of 85%, while the thermally-treated juices were about 81–83%. The 'Blanch-Past' value was slightly higher, although not significantly so (Table 3).

The results from the DPPH• quenching test gave a higher difference of scavenging effect between 'NT' (49%) and thermally-treated samples (about 43%).

These results were completely in contrast to the AA data. It was evident that the RSA cannot be in a simple relationship to the amount of the phenolic antioxidant substances previously listed. The only clear relationship found between RSA and juice composition was with the amount of ascorbic acid (Table 1).

4. Conclusion

The thermally-induced antioxidant and free radical scavenging activities of blood orange juices showed opposite trends in relation to the assay. In particular, the inhibition of enzymatically-mediated linoleic acid peroxidation was increased by thermal treatments, while the scavenging effect toward OH•, generated by Fenton reaction, and DPPH•, decreased.

The first point is sustained by the amounts of some phenolic substances with antioxidant action (anthocyanins and hydroxycinnamates). The increase of these components in thermally treated substrates is known in other vegetables such as carrot, rosemary and sage (Gazzani et al., 1998; Pizzocaro, 1989; Pizzocaro, Gasparoli, & Babbini, 1991; Pizzocaro et al., 1995, 1997). This phenomenon was probably caused by two concomitant events: the thermally induced extraction of antioxidant molecules previously complexed or polymerized and the retention of active principles caused by the inactivation of the enzymes involved in their catabolism.

As for the second point, it was evident that the thermal treatments induced a decrease in the free radical-scavenging activity and were contemporarily responsible for the degradation of ascorbic acid in blood orange juice.

It is clear that the results of the different antioxidant tests were supported by different composition profiles of thermally-treated samples. On the basis of these findings, one could speculate that the phenolic compounds (anthocyanins and hydroxycinnamates) were able to protect against the oxidation of lipophilic substances and ascorbic acid, a water-soluble component, acts as an OH• or DPPH• scavenger.

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