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Assessment of antioxidant activity of carotenoid-enriched extracts from peach fruits using the new LOX/RNO method

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Abbreviations: AA, antioxidant activity; AAPH, 2,2'-azobis(2-amidinopropane); ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate); AUC, area under curve; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; FRAP, Ferric Reducing Antioxidant Power; fluorescein, 3',6'-dihydroxyspiro[isobenzofuran-1[3*H*], 9'[9*H*]-xanthen]-3-one; f.w., fresh weight; LOX, lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12); ORAC, Oxygen Radical Absorbance Capacity; RNO, 4-nitroso-*N,N*-dimethylaniline; TEAC, Trolox Equivalent Antioxidant Capacity; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

Key words: antioxidant activity, carotenoids, LOX/RNO method, peach.

Abstract: Peach (*Prunus persica* L.) fruits contain several health-promoting phytochemicals. Among these, carotenoids, in addition to being involved in determining flesh color, play a relevant role in cell protection against oxidative stress. Nevertheless, antioxidant activity (AA) of peach carotenoids so far has not been investigated in as much detail as phenols. In the present study, for the first time, AA of peach carotenoid extracts was evaluated using the innovative lipoxygenase/4-nitroso-*N,N*-dimethylaniline (LOX/RNO) method, able to simultaneously detect different antioxidant mechanisms and synergistic antioxidant interactions, as well as using the well-known ORAC and TEAC assays. In particular, extracts were obtained from fruits collected in S4 development stage from two yellow-fleshed (Armking and Redhaven) and three white-fleshed (Silverking, Caldesi 2000, IFF331) varieties. The LOX/RNO method gave high AA values (10-150 µmol eq. Trolox/g f.w.), about 85-1900-fold higher than ORAC and TEAC methods. Moreover, the ratio between AA values, measured by the LOX/RNO method, of yellow- and white-fleshed peaches resulted equal to 14, but only 2.6 and 3.6 for ORAC and TEAC, respectively. Results of this study indicate that the LOX/RNO method, measuring high AA values and easily discriminating among samples, is an advisable tool to assess the AA of the carotenoid component in peach.

1. Introduction

Peach (*Prunus persica* L.), a member of the Rosaceae family, is one of the most economically important fruit species in the world, with Italy being the second world producer after China and the first in Europe (FAOSTAT, 2012). The flesh color of the fruit, white or yellow, is an important characteristic driving consumer choice and thus represents a key breeding trait for this crop. From a genetic point of view, it is controlled by a single *locus* and the white phenotype is fully dominant over the yellow (Connors, 1920; Falchi *et al*., 2013). From a biochemical point of view, yellow flesh color is determined by the accumulation of carotenoids in chromoplasts. In particular, in the early stages of the fruit developmental cycle, both white- and yellow-fleshed peaches have very high concentrations of total carotenoids, although masked by the green due to the presence of chlorophyll (Ma *et al*., 2014). In the later stages of the fruit cycle, carotenoids are massively degraded in the white-fleshed, but not in the yellow-fleshed fruits, which keep accumulating these compounds and whose content peaks at full fruit ripening (Ma *et al*., 2014). Recent studies carried out on peach chimeric mutants for flesh color showed a strongly reduced expression of *ccd4*, the gene codifying for dioxygenase in the yellow-fleshed genotype compared to its white-fleshed mutant,

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suggesting a key role of this gene in controlling carotenoid accumulation, hence flesh color in peach (Brandi *et al*., 2011; Adami *et al*., 2013).

In addition to their role in peach flesh color, carotenoids also represent relevant biologically active compounds, responsible for some beneficial health properties associated to increased consumption of fruit and vegetables (Liu, 2003 and references therein; Schreiner and Huyskens-Keil, 2006). In particular, β-carotene, α-carotene and β-cryptoxanthin have an essential function in human nutrition as they are a primary dietary source of provitamin A, which humans are unable to synthesize *de novo*. Moreover, the antioxidant properties of carotenoids, that may act as free-radical scavengers, singlet oxygen quenchers, and lipid antioxidants, have been reported to be highly involved in reducing the risk of cardiovascular disease, cancer, diabetes and other degenerative diseases associated with oxidative stress (Rao and Rao, 2007; Jomova and Valko, 2013).

In recent years several studies have been performed to investigate the antioxidant properties of peach fruit due to carotenoids, as well as anthocyanins and other phenolic compounds; nevertheless, the role of carotenoids so far has not been completely clarified. Peach carotenoid content has been quantified in these reports, as well as antioxidant activity (AA) evaluated using assays measuring *i*) the peroxyl radical scavenging capacity (Oxygen Radical Absorbance Capacity, ORAC) (Campbell and Padilla-Zakour, 2013), *ii*) the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation scavenging capacity (Trolox Equivalent Antioxidant Capacity, TEAC) (Dalla Valle *et al*., 2007; Di Vaio *et al*., 2008; Legua *et al*., 2011; García-Parra *et al*., 2011; Oliveira *et al*., 2012), *iii*) the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (Gil *et al*., 2002; Lavelli *et al*., 2009; Puerta-Gomez and Cisneros-Zevallos, 2011; Zielinski *et al*., 2014), and *iv*) the Fe^{3+} ion reducing capacity (Ferric Reducing Antioxidant Power, FRAP) (Gil *et al*., 2002; Remorini *et al*., 2008; Tavarini *et al*., 2008; Durst and Weaver, 2013; Zielinski *et al*., 2014). In most of these studies, AA was evaluated by studying methanol extracts. Only in very few reports, the AA of both water- and fat-soluble antioxidant fractions (obtained from fruit extraction using water and acetone or THF or ethyl acetate, respectively) was investigated separately (Dalla Valle *et al*., 2007; Di Vaio *et al*., 2008; Legua *et al*., 2011). This was made possible by using only the TEAC assay, measuring mainly the antioxidant reducing capacity; interestingly, generally lower AA values of the lipophilic antioxidant fraction compared to those of the hydrophilic component were obtained, positively correlated to total carotenoid content (Legua *et al*., 2011).

In the present study, AA of extracts highly enriched in carotenoids obtained from the flesh of peach fruits was evaluated for the first time using the advanced and innovative lipoxygenase/4-nitroso-*N,N*-dimethylaniline (LOX/RNO) method (Pastore *et al*., 2009). This is a recently developed AA assay based on the RNO bleaching reaction due to some radical species generated by secondary anaerobic reactions catalyzed by soybean LOX-1 isoenzyme, occurring when the main aerobic cycle of linoleic acid (LH) hydroperoxidation has consumed oxygen (Pastore *et al*., 2000 a) (Fig. 1). With respect to the majority of AA assays, the LOX/RNO method is able to detect the scavenging capacity of antioxidants towards different physiological and biologically relevant radical species, such as alkoxyl ($LO^{\bullet}$), peroxyl ($LOO^{\bullet}$), hydroxyl ($^{\bullet}OH$) and perhaps alkylic ($L^{\bullet}$) radicals, as well as singlet oxygen ($^1O_2$), but this latter only in the presence of imidazole (Pastore *et al*., 2000 a; 2009). Moreover, other important antioxidant functions may be simultaneously detected, including chelating or reducing activities of iron ions essential for the catalysis and generation of radical species, as well as any possible inhibition of the apo-enzyme (Pastore *et al*., 2009) (Fig. 1). Consistently, the LOX/RNO method has been found to highlight very well synergistic effects among antioxidants (Pastore *et al*., 2009; Laus *et al*., 2012 b; Laus *et al*., 2013 b). To date, the LOX/RNO method has been applied to assess AA of food-grade antioxidants (Laus *et al*., 2013 b) and of whole flour of some cereals (Flagella *et al*., 2006; Pastore *et al*., 2009; Laus *et al*., 2012 b), pseudocereals (Laus *et al*., 2012 a), and grain-derived products (Laus *et al*., 2013 a), showing a much higher performance with respect to other commonly used AA assays.

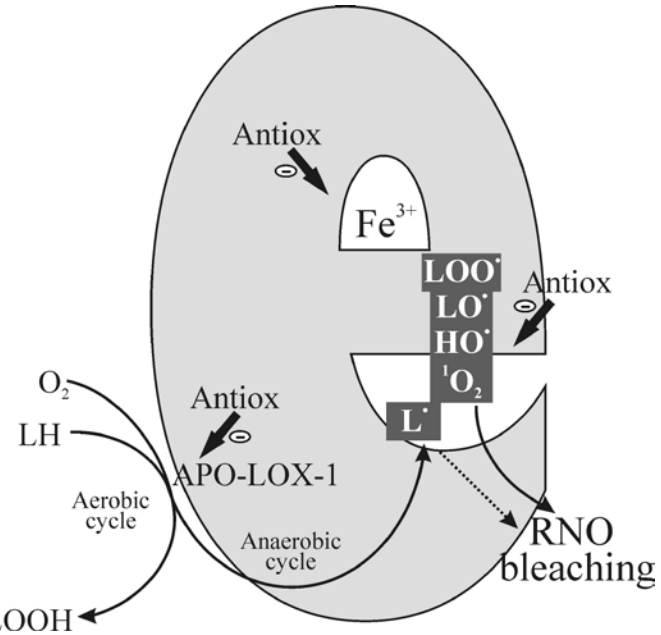

Fig. 1 - Schematic representation of the aerobic and anaerobic cycles catalyzed by the soybean lipoxygenase (LOX)-1 isoenzyme involved in the bleaching of the 4-nitroso-*N,N*-dimethylaniline (RNO). The soybean LOX-1 apoenzyme is represented as e-shaped and Fe^{3+} indicates non-heme iron atom essential for the LOX-1 catalysis. The aerobic cycle of soybean LOX-1-mediated hydroperoxidation of linoleic acid (LH) is schematized, as well as secondary anaerobic reactions involving generation of radical species, able to induce the RNO bleaching. The short black arrows indicate some different mechanisms by which antioxidant compounds may inhibit the LOX-1-dependent RNO bleaching. Antiox, antioxidant compounds; $LO^{\bullet}$, $LOO^{\bullet}$, $L^{\bullet}$, alkoxyl, peroxyl and alkylic radicals of LH; LOOH, 13-hydroperoxy derivative of LH; $^1O_2$, singlet oxygen; $^{\bullet}OH$, hydroxyl radical.

The objective of this study was to evaluate the performance of the LOX/RNO method with respect to AA assessment of the carotenoid fraction extracted from the flesh of peach fruits of some yellow and white varieties. To do this, the LOX/RNO method was compared with two well-established methodologies for AA measurement: the TEAC (Re *et al*., 1999) and ORAC (Ou *et al*., 2001) assays.

2. Materials and Methods

Chemicals

All reagents at the highest commercially available purity were purchased from Sigma-Aldrich Corp. (St. Louis, Mo., U.S.A.).

Plant material

Fruit from yellow-fleshed (Armking and Redhaven) and white-fleshed (Silverking, Caldesi 2000 and IFF331) cultivars/breeding selections was harvested from trees grown in the experimental farm of CRA-FRU (Roma, Italy) and CRA-FRF (Forlì, Italy). Replicates of three representative fruits at stage S4 (full ripening phase) of fruit development were sampled, peeled, cut into 0.5-cm slices and the mesocarp was immediately frozen in liquid nitrogen and stored at -80°C.

Preparation of aqueous solutions of linoleate and β-carotene

The sodium linoleate solution was prepared as described in Pastore *et al*. (2000 b; 2009) and the exact linoleate concentration was determined by means of the LOX assay (Pastore *et al*., 2000 b), by using a Perkin-Elmer λ45 UV-Vis Spectrophotometer (Perkin-Elmer, Wellesley, MA), managed by UV WINLAB software (Perkin Elmer version 2.85.04). The β-carotene solution was prepared according to the method reported by Pastore *et al*. (2000 b; 2009), and the concentration was spectrophotometrically determined as described in Pastore *et al*. (2000 b).

Extraction of carotenoids from peach fruit

Extraction was carried out according to the procedure described in Fraser *et al*. (2000) with some modifications. All manipulations were carried out on ice and shielded from strong light. Freeze-dried material (about 3.5 and 5 g for white- and yellow-fleshed fruits, respectively) was finely ground into a powder with a mortar and pestle using liquid nitrogen. Methanol was added according to a (v/w) ratio equal to 7.5 mL/g of ground tissue; the suspension was mixed by inversion and incubated for 5 min in an ice-water bath. Then, 50 mM Tris-HCl buffer pH 7.5 (containing 1 M NaCl) was added to the mixture according to a (v/w) ratio equal to 7.5 mL/g of homogenized tissue and incubated in an ice-water bath for 10 min. Chloroform at a (v/w) ratio equal to 2 mL/g of tissue was added to the mixture, incubated on ice for 10 min and then centrifuged at 5000x*g* for 5 min at 4°C. The aqueous phase was removed and the chloroform hypophase was pooled. Determination of total carotenoid content was performed on chloroform extract as described in the next section. Then, the chloroform extract was partitioned in two volumes. For AA measurements with the TEAC and ORAC methods, an aliquot was evaporated to dryness under vacuum at 40°C using a Buchi evaporator and the dry residue was reconstituted in absolute ethanol. As for the LOX/RNO assay, the second aliquot of the chloroform extract was added with Tween 80 according to a ratio equal to 0.4 µL Tween 80/µg of carotenoids; the mixture was dried under vacuum at 40°C and the dry residue was reconstituted in 100 mM sodium borate buffer pH 9.0.

Spectrophotometric determination of total carotenoids of extracts from peach fruits

Quantification of total carotenoids was carried out according to the procedure described by Lichtenthaler (1987) and by Lichtenthaler and Wellburn (1983). Briefly, the absorbance spectra of appropriate dilutions in 80% (v/v) acetone of the chloroform extract (see previous section) was recorded in the VIS region. Carotenoid concentration was calculated by means of a proper equation using a specific absorption coefficient at 470 nm of 198 $mL \cdot mg^{-1} \cdot cm^{-1}$, and involving a correction for chlorophyll a and b content based on absorbance measurements at 663 and 647 nm.

Determination of Antioxidant Activity (AA) by the LOX/RNO, ORAC and TEAC methods

The LOX/RNO reaction was spectrophotometrically monitored, as described in Pastore *et al*. (2000 a; 2009), by measuring the RNO absorbance decrease at 440 nm and 25°C in a reaction mixture (2 mL) containing 100 mM sodium borate buffer pH 9.0, 1 mM sodium linoleate, 1.5 µL Tween 20/µmol linoleate and 15 µM RNO; the reaction was started by adding 0.3 Enzymatic Units (EU) of soybean LOX-1. Since carotenoid extracts reconstituted in sodium borate buffer containing 0.4 µL Tween 80/µg carotenoids were analyzed, all LOX/RNO measurements were carried out in the presence of a constant volume (0.5 µL/mL) of Tween 80 in the assay mixture. The LOX/RNO reaction was measured both in the absence (control) and presence of carotenoid extract (or ±-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Trolox, used as a standard antioxidant). The (%) decrease of the rate of RNO bleaching measured in the presence of extract (or Trolox) with respect to the rate of the control reaction was used to quantify AA. This was done by means of a dose-response curve derived for Trolox by plotting the (%) decrease of the rate of RNO bleaching as a function of standard antioxidant concentration. In particular, to calculate the rate of the LOX/RNO reaction in the presence of extract, the LOX-1-depedent bleaching of carotenoids was also monitored by measuring the absorbance decrease at 440 nm in the above reported assay mixture lacking RNO. Then, a new trace was built, representing the difference between the trace of the LOX/RNO reaction in the presence of carotenoid extract and that relative to carotenoid bleaching

(for details see Results).

The ORAC protocol, described in Ou *et al*. (2001) and modified as in Pastore *et al*. (2009) and Laus *et al*. (2012 b), was applied. Fluorescence intensity decay due to 3',6'-dihydroxyspiro[isobenzofuran-1[3*H*], 9'[9*H*]-xanthen]-3-one (fluorescein) oxidation by peroxyl radicals generated by 2,2'-azobis(2-amidinopropane) (AAPH) thermal decomposition was continuously monitored at 37°C at excitation and emission wavelengths of 485 and 515 nm, respectively. To quantify AA, the area under the fluorescence decay kinetic curve (area under curve, AUC) was used and, in particular, the net AUC (AUC_{net}), obtained by subtracting AUC of the blank from that of the sample. AA was calculated by means of a proper dose-response curve prepared with Trolox by plotting the AUC_{net} as a function of standard antioxidant concentration. Since carotenoid extracts reconstituted in ethanol were used, all ORAC measurements were carried out in the presence of a constant volume of ethanol in the assay mixture.

The TEAC protocol, reported in Re *et al*. (1999) and modified as in Pastore *et al*. (2009) and Laus *et al*. (2012 b), was used. The coloured radical cation $ABTS^{\bullet+}$ was produced by ABTS oxidation with potassium persulfate solution. Absorbance at 734 nm and 25°C (A_{734}) was measured after a fixed time of incubation of carotenoid extract (or Trolox) with the $ABTS^{\bullet+}$ solution (diluted in absolute ethanol). The (%) decrease of A_{734} measured after 3 min incubation of extract (or Trolox), with respect to A_{734} of the uninhibited radical cation solution, was calculated; AA was quantified by means of a proper concentration-response curve prepared with Trolox by plotting the (%) decrease of A_{734} as a function of standard antioxidant concentration.

For all three methods, determinations were carried out in triplicate by analyzing at least three different amounts of extract. A linear dependence of the inhibition on the amount of extract was verified by linear regression analysis of data. Then, AA was obtained by comparing the slope derived by linear regression analysis with that of the calibration curve prepared with Trolox.

Statistical analysis

Distribution of data from figure 4 was evaluated using the Shapiro-Wilk and Jarque-Bera tests, and homogeneity of variances was verified by the Bartlett's test. Figure 4 data were submitted to a "one-factor" analysis of variance (ANOVA) and the mean separation was tested by the Duncan's test at 0.01 *P* level of significance. Statistical analysis was performed using Statistica (data analysis software system), version 7 (StatSoft, Tulsa, Oklahoma, USA).

3. Results

Application of the LOX/RNO method to AA assessment of extracts showing high ß-carotene concentration is complicated both by the high absorbance of ß-carotene in the visible light region and by the ability of LOX to cause carotenoid bleaching. Thus, a preliminary investigation regarding this point was carried out.

In figure 2 the absorbance spectra of both RNO and β-carotene aqueous solutions are reported, as recorded in the visible region of the electromagnetic spectrum: a large overlapping of both spectra in the entire investigated visible region is clearly evident, as well as a high absorbance value of ß-carotene at 440 nm, representing the wavelength of the RNO absorption maximum. In light of these absorbance properties of carotenoids, the LOX-1-catalyzed RNO bleaching cannot be easily monitored at 440 nm.

To overcome these problems, in this study a new simple LOX/RNO protocol was developed for AA assessment of carotenoid-enriched extracts. Figure 3 shows evaluation according to the new protocol of the LOX-1-dependent RNO bleaching in the presence of carotenoids extracted from the yellow-fleshed peach fruits of cv. Armking. In particular, in figure 3A a typical experimental trace of the LOX/RNO control (in the absence of extract) reaction is reported: it was spectrophotometrically monitored by continuously measuring the RNO absorbance decrease at 440 nm and it consists of a lag phase (representing the time occurring to consume oxygen in the reaction mixture due to the primary LOX-1 reaction of linoleate hydroperoxidation), and a true RNO bleaching phase (due to the radical species generated by LOX-1 when anaerobiosis is approached in the assay mixture), occurring, in this experiment, at a rate equal to about 0.15 $\Delta A_{440\ nm} \cdot min^{-1}$. In figure 3B the trace relative to the RNO bleaching in the presence of 1.13 µg carotenoids from Armking (RNO *plus* carotenoid bleaching, trace a) is shown. In the same figure, the trace b is reported, representing the experimental curve relative to carotenoid bleaching reaction, obtained

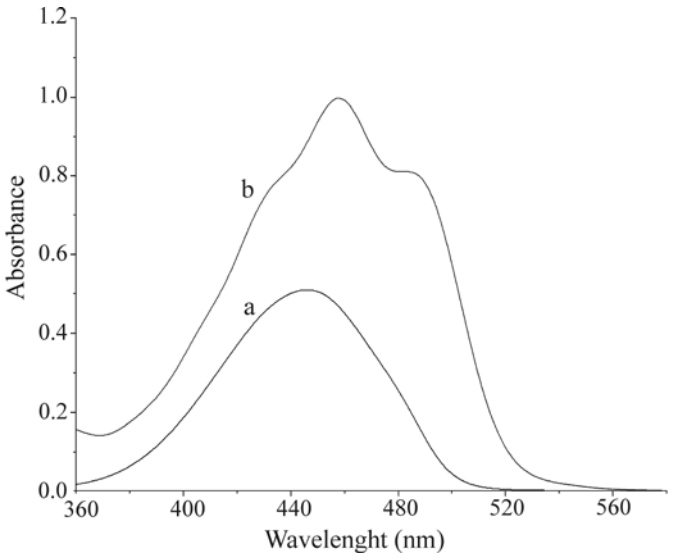

Fig. 2 - RNO (a) and ß-carotene (b) absorbance spectra. Aqueous solution of ß-carotene was prepared as reported in Methods. Absorbance spectra of 15 µM RNO (trace a) and 8 µM ß-carotene (trace b) were recorded in 2 mL of 100 mM sodium borate buffer pH 9.0.

by measuring the absorbance decrease at 440 nm in the absence of RNO: it clearly shows carotenoid absorbance decrease due to oxidation by radicals produced during secondary anaerobic reactions associated to the LOX-1-mediated linoleate hydroperoxidation (Barimalaa and Gordon, 1988; Pastore *et al.*, 2000 a). Moreover, the curve representing the difference between traces a and b, obtained by mathematical processing of these curves using a specific software, is also reported (trace indicated as "a *minus* b"), showing a rate equal to about 0.12 $\Delta A_{440\ nm} \cdot min^{-1}$. This newly built curve only reflects the effect of carotenoids on RNO bleaching, excluding any absorbance decrease due to LOX-1-dependent carotenoid bleaching; thus, its rate may be compared to that of the LOX/RNO control reaction. The comparison of the slopes of the trace "a *minus* b" and the control one shows that the amount of extract from cv. Armking containing 1.13 µg of carotenoids is able to induce an about 25% decrease of the rate of RNO bleaching. In the same experiment, an increasing inhibition of the reaction rate with increasing amount of Armking carotenoid extract, evaluated according to the same protocol, was also observed. In particular, a linear dependence between inhibition and amount of extract was found in the studied 0.75-2.25 µg carotenoid range (Fig. 3D). In figure 3C the Trolox-dependent inhibition of the rate of RNO bleaching as a function of the standard antioxidant concentration is reported, showing a linear dependence of the inhibition in the 20-50% range on Trolox concentration ranging from 2 to 7.5 mM, described by the equation: y(inhibition) = 6.059x(concentration millimolar of

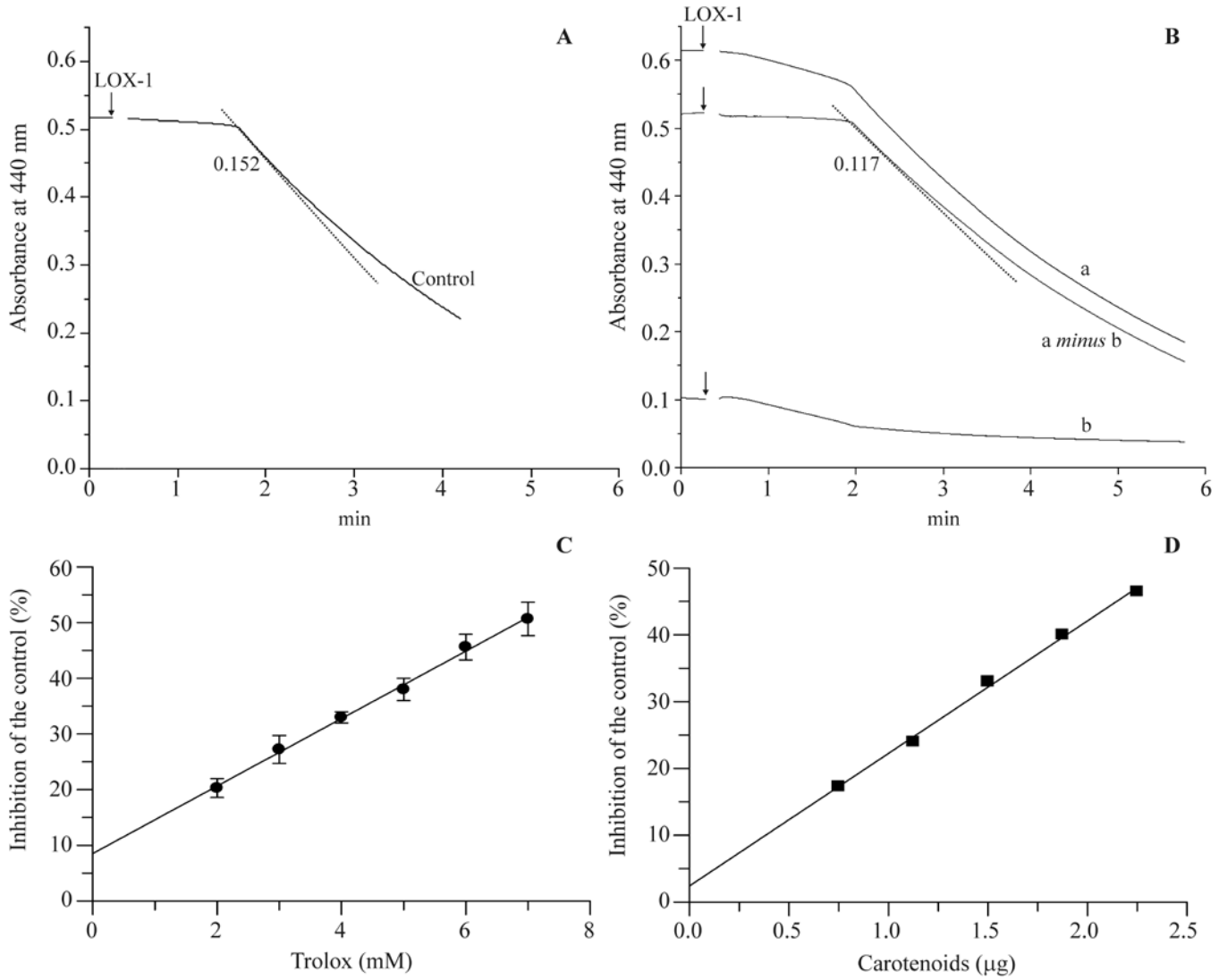

Fig. 3 - The LOX-1-dependent RNO bleaching in the absence (A, control) and presence (B) of carotenoid-enriched extract from peach fruit and linear dependence of the inhibition on Trolox concentration (C) and extract amount (D). The LOX/RNO reaction was monitored as described in Methods in the absence (A, control) and in the presence of 1.13 µg carotenoids extracted from the yellow-fleshed cv. Armking (B, trace a). In (B) the LOX-1-dependent bleaching of the same amount (1.13 µg) of carotenoid extract was also reported (trace b), as well as the curve obtained from the difference between traces a and b (trace "a *minus* b"). In (C) the calibration curve obtained with Trolox is reported; in (D) the (%) inhibition of the rate of the LOX/RNO reaction is reported as a function of extract amount, expressed as carotenoid content. The rates of the RNO bleaching, calculated as the highest slopes (dotted lines) to the experimental curves, are reported, expressed as $\Delta A_{440\ nm} \cdot min^{-1}$. For AA calculation see the text.

Trolox) + 8.505 (r=0.999, $P \leq 0.001$). By means of this Trolox-based calibration curve, the AA value for Armking extract was calculated, resulting, in this experiment, equal to 120±6 µmol eq. Trolox/g fresh weight. The new developed protocol was also applied to measure the AA of carotenoid extract obtained from the white-fleshed cv. Silverking. In this case, a linear dependence between inhibition and amount of extract was obtained in the studied 0.075-0.2 µg carotenoid range; the inhibition corresponded, in that experiment, to an AA value equal to 7.5±2 µmol eq. Trolox/g fresh weight. Interestingly, the ratio between AA values of the yellow-fleshed Armking and the white-fleshed Silverking extracts resulted equal to 16, very similar to the ratio (about 17) between carotenoid content of the extracts (13.5 and 0.8 µg/g fresh weight, respectively, in these experiments).

The study was extended to three other peach varieties, one yellow-fleshed (Redhaven) and two white-fleshed (Caldesi 2000 and IFF331). In Table 1, AA values of carotenoid extracts obtained from all tested genotypes, measured by means of the LOX/RNO, are reported and compared with those obtained by using the ORAC and TEAC assays. The LOX/RNO method provided AA values ranging from 10 (white-fleshed genotypes) to 150 µmol eq. Trolox/g fresh weight (yellow-fleshed genotypes). In Table 1 the carotenoid content of extracts is also reported for all genotypes under study, ranging from 0.25 (white-fleshed genotypes) to 18.5 µg/g fresh weight (yellow-fleshed genotypes). Interestingly, a highly statistically significant positive correlation was obtained between AA values provided by the LOX/RNO method and carotenoid content (r = 0.996, $P \leq 0.001$). AA values obtained by the TEAC assay also resulted significantly correlated to carotenoid content (r = 0.997, $P \leq 0.001$); a lower correlation was obtained for the ORAC protocol (r = 0.824, $P \leq 0.001$); high correlations were also obtained between AA values measured by the LOX/RNO method and that obtained using the other assays (LOX/RNO-TEAC r = 0.992, $P \leq 0.001$; LOX/RNO-ORAC r = 0.837, $P \leq 0.001$; ORAC-TEAC r = 0.839, $P \leq 0.001$).

Figure 4 presents the ratio between the yellow-fleshed cultivars and white-fleshed genotypes under study in terms of carotenoid content, as well as the ratio between AA mean values of yellow- and white-fleshed peach fruits, as evaluated by means of the LOX/RNO, ORAC and TEAC methods. The yellow-fleshed genotypes have a carotenoid content on average 36 times higher than that of white-fleshed varieties. Interestingly, the AA mean value obtained by the LOX/RNO method for the yellow-fleshed genotypes resulted about 14 times higher than those measured for the white-fleshed varieties. The same ratios $AA_{\text{yellow-fleshed fruits}}/AA_{\text{white-fleshed fruits}}$ were only about 2.6 and 3.6 in the case of the ORAC and TEAC assays, respectively.

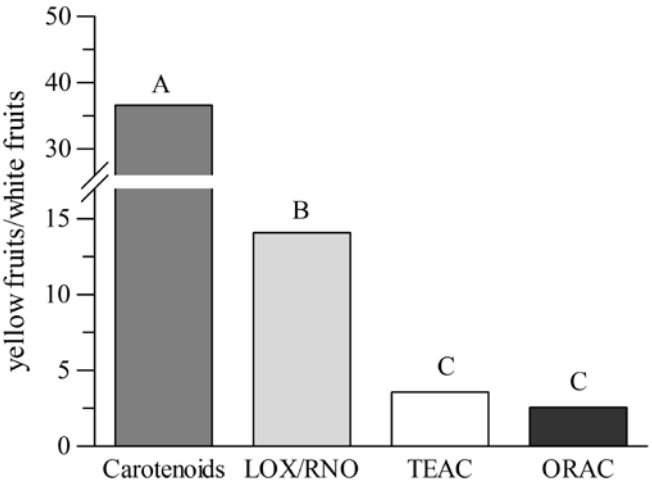

Fig. 4 - Ratio between yellow- and white-fleshed peach fruits in terms of carotenoid content and antioxidant activity, evaluated by means of the LOX/RNO, ORAC and TEAC methods. Data are reported as mean value (n=3 different experiments). Different capital letters indicate significant differences at 0.01 *P* level, according to the Duncan's test.

4. Discussion and Conclusions

In this study, the LOX/RNO method was applied for the first time to assess antioxidant properties of peach fruits, in

Table 1 - Antioxidant Activity (AA), evaluated by means of the LOX/RNO, ORAC and TEAC methods, and carotenoid content of extracts from yellow- and white-fleshed peach genotypes

Genotype	Carotenoid content (µg/g f.w.)	AA (µmol eq. Trolox/g fresh weight)		
		LOX/RNO	ORAC	TEAC
Yellow-fleshed				
Redhaven	18.33±0.75	155.3 ± 10.3	0.325 ± 0.042	0.082 ± 0.002
Armking	14.11±0.71	125.3 ± 9.5	0.444 ± 0.063	0.068 ± 0.001
White-fleshed				
Silverking	0.75 ± 0.08	9.6 ± 3.5	0.211 ± 0.039	0.025 ± 0.001
Caldesi 2000	0.34 ± 0.02	10.5 ± 1.2	0.112 ± 0.007	0.018 ± 0.0001
IFF331	0.24 ± 0.01	9.8 ± 1.5	0.129 ± 0.006	0.020 ± 0.0001

All data refer to fresh weight and are reported as mean value ± standard deviation (n=3 different experiments).

comparison with the widely used ORAC and TEAC assays. In particular, the carotenoid component was studied by comparing some yellow- and white-fleshed genotypes. Unfortunately, application of the LOX/RNO method to AA determination of carotenoid extracts from peach fruits is strongly affected by absorbance properties of carotenoids, whose absorbance spectra largely overlap the one of RNO. Moreover, it should be considered that carotenoids are also subjected to absorbance bleaching due to their co-oxidation associated to the LOX-mediated hydroperoxidation of polyunsaturated fatty acids (Barimalaa and Gordon, 1988; Pastore *et al*., 2000 a). In a previous study, in order to evaluate the effect of ß-carotene on the LOX/RNO reaction, these problems were overcome by measuring the RNO absorbance changes as absorbance difference ($\Delta_{394nm-512nm}$) using a double-wavelength spectrophotometer, an expensive and not widespread laboratory instrumentation (Pastore *et al*., 2009). Interestingly, using that experimental approach, a very high sensitivity to ß-carotene of the LOX/RNO reaction was found, with an IC_{50} value in the micromolar range (Pastore *et al*., 2009), consistent with the inhibition by ß-carotene of LOXes from different sources (Lomnitski *et al*., 1993).

In this paper, to evaluate antioxidant properties of carotenoids by means of the LOX/RNO reaction, a new experimental approach was developed involving the use of a simple spectrophotometer, which is generally found in research laboratories. This is a multi-step protocol involving the following spectrophotometric measurements: *i*) the LOX-1-depedent RNO bleaching in the absence of carotenoid-enriched extract (control reaction); *ii*) the RNO bleaching in the presence of carotenoids (RNO *plus* carotenoid bleaching reaction); *iii*) the carotenoid bleaching (at 440 nm) in the absence of RNO (carotenoid bleaching reaction); *iv*) mathematical processing by specific software of the traces obtained at points *ii*) and *iii*) so as to build a new curve in which the absorbance value at each time is represented by the difference between the respective values of the traces *ii*) and *iii*). Excluding absorbance decrease due to LOX-1-dependent carotenoid bleaching, the rate of the new curve reflects only the effect of carotenoids on RNO bleaching, making it possible to compare the rate of the LOX/RNO control reaction (point *i*), and allowing calculation of the (%) decrease of rate of RNO bleaching and, finally, AA quantification by means of a dose-response curve prepared with Trolox.

To validate the newly developed protocol, measurement of the AA of carotenoid extract obtained from the yellow-fleshed peach fruits of cv. Armking was undertaken, as well as from the white-fleshed cv. Silverking. In both cases, the inhibition of the reaction rate was found to be linearly dependent on the amount of carotenoid extract; the inhibitions measured in these experiments allowed calculation of an AA value of the yellow-fleshed Armking fruits about 15-fold higher than that obtained for the white-fleshed Silverking extract. Interestingly, extract from Armking fruits showed a carotenoid content about 16-fold higher than that measured for Silverking. These data confirm the suitability of the modified LOX/RNO protocol to measure AA of carotenoid compounds.

The study was extended to carotenoid extracts obtained from other yellow- and white-fleshed peach varieties. As already observed in previous studies (Pastore *et al*., 2009; Laus *et al*., 2012 a, b; Laus *et al*., 2013 a, b), the method LOX/RNO provided very high AA values (10-150 µmol eq. Trolox/g fresh weight) which were much higher than those measured by ORAC and TEAC assays (about 85-350-fold and 530-1900-fold higher, respectively) and they showed a statistically significant positive correlation with carotenoid content, higher than that obtained with the other assays in comparison.

Moreover, the ratio between AA values measured by the LOX/RNO method of yellow- and white-fleshed peaches resulted much higher than that obtained using the ORAC and TEAC assays, thus indicating the capability of the newly developed LOX/RNO protocol to highlight much higher differences among the different tested samples with respect to the other compared AA assays.

On the whole, the results of this paper indicate that the LOX/RNO method, applied according to the new protocol developed in this study, is able to measure high AA values of carotenoid-enriched extracts from peach fruits, highly related to carotenoid content, and to easily discriminate among samples. Although TEAC and ORAC values also show correlation with carotenoid content, they measure low AA and few differences among yellow- and white-fleshed varieties. In conclusion, the LOX/RNO method may represent a recommended tool to assess AA of the carotenoid component in peach fruits. Therefore, the use of this assay in studies regarding AA measurements of other fruits and vegetables is worthwhile.

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References

ADAMI M., DE FRANCESCHI P., BRANDI F., LIVERANI A., GIOVANNINI D., ROSATI C., DONDINI L., TARTARINI S., 2013 - *Identifying a carotenoid cleavage dioxygenase (ccd4) gene controlling yellow/white fruit flesh color of peach.* - Plant Mol. Biol. Rep., 31: 1166-1175.

BARIMALAA I.S., GORDON M.H., 1988 - *Cooxidation of β-carotene by soybean lipoxygenase.* - J. Agric. Food Chem., 36(4): 685-687.

BRANDI F., BAR E., MOURGUES F., HORVÁTH G., TURCSI E., GIULIANO G., LIVERANI A., TARTARINI S., LEWINSOHN E., ROSATI C., 2011 - *Study of "Redhaven" peach and its white-fleshed mutant suggests a key role of CCD4 carotenoid dioxygenase in carotenoid and norisoprenoid volatile metabolism.* - BMC Plant Biol., 11:24 doi:10.1186/1471-2229-11-24.

CAMPBELL O.E., PADILLA-ZAKOUR O.I., 2013 - *Phenolic and carotenoid composition of canned peaches (*Prunus persica*) and apricots (*Prunus armeniaca*) as affected by variety and peeling.* - Food Res. Int., 54(1): 448-455.

CONNORS C.H., 1920 - *Some notes on the inheritance of unit characters in the peach.* - P. Am. Soc. Hortic. Sci., 16: 24-36.

DALLA VALLE A.Z., MIGNANI I., SPINARDI A., GALVANO F., CIAPPELLANO S., 2007 - *The antioxidant profile of three different peaches cultivars (*Prunus persica*) and their short-term effect on antioxidant status in human.* - Eur. Food Res. Technol., 225(2): 167-172.

DI VAIO C., GRAZIANI G., MARRA L., CASCONE A., RITIENI A., 2008 - *Antioxidant capacities, carotenoids and polyphenols evaluation of fresh and refrigerated peach and nectarine cultivars from Italy.* - Eur. Food Res. Technol., 227(4): 1225-1231.

DURST R.W., WEAVER G.W., 2013 - *Nutritional content of fresh and canned peaches.* - J. Sci. Food Agric., 93(3): 593-603.

FALCHI R., VENDRAMIN E., ZANON L., SCALABRIN S., CIPRIANI G., VERDE I., VIZZOTTO G., MORGANTE M., 2013 - *Three distinct mutational mechanisms acting on a single gene underpin the origin of yellow flesh in peach.* - Plant J., 76(2): 175-187.

FLAGELLA Z., TOZZI D., SOCCIO M., TARANTINO E., PASTORE D., 2006 - *Grain antioxidant activity in different herbaceous crop species.* - Fragmenta Agronomica, 11: 753-754.

FRASER P.D., PINTO M.E.S., HOLLOWAY D.E., BRAMLEY P.M., 2000 - *Application of high-performance liquid chromatography with photodiode array detection to the metabolic profling of plant isoprenoids.* - Plant J., 24(4): 551-558.

GARCÍA-PARRA J., GONZÁLEZ-CEBRINO F., DELGADO J., LOZANO M., HERNÁNDEZ T., RAMÍREZ R., 2011 - *Effect of thermal and high-pressure processing on the nutritional value and quality attributes of a nectarine purée with industrial origin during the refrigerated storage.* - J. Food Sci., 76(4): C618-C625.

GIL M.I., TOMÁS-BARBERÁN F.A., HESS-PIERCE B., KADER A.A., 2002 - *Antioxidant capacities, phenolic compounds, carotenoids and vitamin C contents of nectarine, peach and plum cultivars from California.* - J. Agric. Food Chem., 50: 4976-4982.

JOMOVA K., VALKO M., 2013 - *Health protective effects of carotenoids and their interactions with other biological antioxidants.* - Eur. J. Med. Chem., 70: 102-110.

LAUS M.N., DENOTH F., CIARDI M., GIORGETTI L., PUCCI L., SACCO R., PASTORE D., LONGO V., 2013 a - *The antioxidant-rich food supplement Lisosan G induces reversion of hepatic steatosis in mouse.* - Med. Weter., 69(4): 235-240.

LAUS M.N., GAGLIARDI A., SOCCIO M., FLAGELLA Z., PASTORE D., 2012 a - *Antioxidant activity of free and bound compounds in quinoa (*Chenopodium quinoa *Willd.) seeds in comparison with durum wheat and emmer.* - J. Food Sci., 77(11): C1150-C1155.

LAUS M.N., SOCCIO M., PASTORE D., 2013 b - *Evaluation of synergistic interactions of antioxidants from plant foods by a new method using soybean lipoxygenase.* - J. Food Nutr. Res., 52(4): 256-260.

LAUS M.N., TOZZI D., SOCCIO M., FRATIANNI A., PANFILI G., PASTORE D., 2012 b - *Dissection of antioxidant activity of durum wheat (*Triticum durum *Desf.) grains as evaluated by the new LOX/RNO method.* - J. Cereal Sci., 56: 214-222.

LAVELLI V., POMPEI C., CASADEI M.A., 2009 - *Quality of nectarine and peach nectars as affected by lye-peeling and storage.* - Food Chem., 115(4): 1291-1298.

LEGUA P., HERNÁNDEZ F., DÍAZ-MULA H.M., VALERO D., SERRANO M., 2011 - *Quality, bioactive compounds, and antioxidant activity of new flat-type peach and nectarine cultivars: a comparative study.* - J. Food Sci., 76(5): C729-C735.

LICHTENTHALER H.K., 1987 - *Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes.* - Method Enzymol., 148: 350-382.

LICHTENTHALER H.K., WELLBURN A.R., 1983 - *Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents.* - Biochem. Soc. Trans., 11: 591-592.

LIU R.H., 2003 - *Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals.* - Am. J. Clin. Nutr., 78: 517S-520S.

LOMNITSKI L., BAR-NATAN R., SKLAN D., GROSSMAN S., 1993 - *The interaction between β-carotene and lipoxygenase in plant and animal systems.* - Biochim. Biophys. Acta, 1167(3): 331-338.

MA J., LI J., ZHAO J., ZHOU H., REN F., WANG L., GU C., LIAO L., HAN Y., 2014 - *Inactivation of a gene encoding carotenoid cleavage dioxygenase (ccd4) leads to carotenoid-based yellow coloration of fruit flesh and leaf midvein in peach.* - Plant Mol. Biol. Rep., 32: 246-257.

OLIVEIRA A., PINTADO M., ALMEIDA D.P.F., 2012 - *Phytochemical composition and antioxidant activity of peach as affected by pasteurization and storage duration.* - LWT - Food Sci. Technol., 49(2): 202-207.

OU B., HAMPSCH-WOODILL M., PRIOR R.L., 2001 - *Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe.* - J. Agric. Food Chem., 49: 4619-4626.

PASTORE D., LAUS M.N., TOZZI D., FOGLIANO V., SOCCIO M., FLAGELLA Z., 2009 - *New tool to evaluate a comprehensive antioxidant activity in food extracts: bleaching of 4-nitroso-N,N-dimethylaniline catalyzed by the soybean lipoxygenase-1.* - J. Agric. Food Chem., 57: 9682-9692.

PASTORE D., TRONO D., PADALINO L., DI FONZO N., PASSARELLA S., 2000 a - *p-Nitrosodimethylaniline (RNO) bleaching by soybean lipoxygenase-1. Biochemical characterization and coupling with oxodiene formation.* - Plant Physiol. Biochem., 38: 845-852.

PASTORE D., TRONO D., PADALINO L., SIMONE S., VALENTI D., DI FONZO N., PASSARELLA S., 2000 b - *Inhibition by α-tocopherol and L-ascorbate of linoleate hydroperoxidation and β-carotene bleaching activities in durum*

wheat semolina. - J. Cereal Sci., 31: 41-54.

PUERTA-GOMEZ A.F., CISNEROS-ZEVALLOS L., 2011 - *Postharvest studies beyond fresh market eating quality: Phytochemical antioxidant changes in peach and plum fruit during ripening and advanced senescence.* - Postharvest Biol. Tec., 60(3): 220-224.

RAO A.V., RAO L.G., 2007 - *Carotenoids and human health.* - Pharm. Res., 55: 208-216.

RE R., PELLEGRINI N., PROTEGGENTE A., PANNALA A., YANG M., RICE-EVANS C., 1999 - *Antioxidant activity applying an improved ABTS radical cation decolorization assay.* - Free Radical Bio. Med., 26: 1231-1237.

REMORINI D., TAVARINI S., DEGL'INNOCENTI E., LORETI F., MASSAI R., GUIDI L., 2008 - *Effect of rootstocks and harvesting time on the nutritional quality of peel and flesh of peach fruits.* - Food Chem., 110(2): 361-367.

SCHREINER M., HUYSKENS-KEIL S., 2006 - *Phytochemicals in fruit and vegetables: Health promotion and postharvest elicitors.* - Crit. Rev. Plant Sci., 25: 267-278.

TAVARINI S., DEGL'INNOCENTI E., REMORINI D., MASSAI R., GUIDI L., 2008 - *Preliminary characterization of peach cultivars for their antioxidant capacity.* - Int. J. Food Sci. Tech., 43(5): 810-815.

ZIELINSKI A.A.F., ÁVILA S., ITO V., NOGUEIRA A., WOSIACKI G., HAMINIUK C.W.I., 2014 - *The association between chromaticity, phenolics, carotenoids, and in vitro antioxidant activity of frozen fruit pulp in Brazil: an application of chemometrics.* - J. Food Sci., 79(4): C510-C516.