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Antioxidant Properties of Minimally processed (ready-to-eat) Italian *Cichorium* genus salads

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Abstract

The modern lifestyle is characterized by a continuous increase in the demand of ready-to-eat fresh food products subjected to minimal processes prior marketing. Attention is drawn towards the effect of this processing on the antioxidant/antiradical properties of two typical Italian salads belonging to *Cichorium* genus. Four chemical assays (anti-peroxyl radical activity, DPPH• assay, metal chelating ability assay, and reducing power assay) were used for evaluating these properties. The results indicated that the anti-peroxyl radical activity, the anti-DPPH radical activity, the metal chelating ability, and the reducing power were significantly higher in fresh samples as compared to 8-days stored samples ($p<0.001$). Minimal processing applied to vegetables further affected the tested properties causing a decrease over time from 24h to 8d of storage at 4°C. Overall, the results presented in this study showed that any minimal processing applied to ready-to-eat vegetable affects the antioxidant/antiradical properties determining a decrease in the values registered in the four chemical assays.

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1. Introduction

The modern lifestyle is characterized by a continuous increase in the demand of packaged, ready-to-eat products, including fruits and vegetables. These products are fresh food subjected to minimal processes, such as washing, peeling, cutting, or slicing, that should be carried out in order both to preserve their freshness and nutritional properties, and also to maintain a sufficient shelf life period for the distribution and consumption of food. However, such simple manipulations can cause undesirable changes in the organoleptic, nutritional and health properties of the products. Negative effects on the texture and the color of vegetable/fruit and the loss of soluble or unstable components could derive from these operations and also causing the microbial contamination. Therefore, the loss of quality and safety of the fresh product could derive from these treatments. In the last decade, a high number of researches had been conducted to study and develop optimized conditions to be applied to ready-to-eat foods to minimize negative changes. In particular, the influence of packaging technique on the microbiota development was extensively investigated [1-5]. Generally, the application of chemical or natural alternative disinfectants, the respect of the cold chain (4°C) and more recently the application of modified atmosphere technology, that contribute to reduce also the chemical degradation of food components, are the most common approaches that have been used to maintain the integrity of the vegetable properties. Because of healthy properties of vegetables due to their nutraceuticals, nowadays they well recognized and considered very important and a high numbers of studies are reported about this topic. The relationship between the presence of substances showing antioxidant activity and the prevention of cardiovascular and chronic diseases are especially studied [6-8]. The salads belonging to *Cichorium* genus showed a number of healthy

effects [9, 10] among which there are good antioxidant activity when tested in chemical systems [11, 12] and protective activity against rat liver microsome lipid peroxidation induced by CCl_4 in an *ex vivo* system [13]. Due to these important healthy properties, plant genetic engineering could act at different metabolic pathway levels, promoting the expression of the gene/s encoding enzymes involved in the synthesis of antioxidant compounds, in order to produce biofortified salad species [14].

However, before applying such biotechnologies, it is important to study the stability of the antioxidant compounds present in the vegetables, especially in the ready-to-eat products.

The aim of this study was (i) to monitor the antioxidant/antiradical properties of the water soluble components extracted from two different types of salads belonging to *Cichorium* genus, i.e. "Treviso tardivo" red chicory (*Cichorium intybus* var. *silvestre*), and "Variegato di Castelfranco" (cross between *C. intybus* var. *silvestre* and *C. endivia* var. *latifolium*) chicory when subjected to minimal processing to obtain ready-to-eat product, and (ii) to investigate the effect on such properties when salads have been stored at 4°C for a long period (8 days) after the manufacture of the product. The antiradical activity was tested against the peroxyl radical and the stable colored 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical; the reducing power and the iron chelating ability of the water soluble fraction of the different samples were also evaluated.

2. Materials and Methods

2.1 Chemicals

Methanol, sodium hydroxide, monobasic potassium phosphate, Tween 20, hydrochloric acid were purchased from Merck (Darmstadt, Germany). β-carotene, linoleic acid, radical DPPH, (±)-6-Hydroxy- 2,5,7,8 tetramethyl chromane-2-carboxylic acid (Trolox C), Iron (II) chloride, ferrozine, ethylene diamine tetraacetic acid (EDTA), potassium hexacyanoferrate (III), iron (III) chloride, ascorbic acid, and chloroform were purchased from Sigma-Aldrich (Steinheim, Germany). All reagents were of analytical grade.

2.2 Vegetable samples

"Treviso tardivo" red chicory (*Cichorium intybus* var. *silvestre*) and "Variegato di Castelfranco" (cross between *C. intybus* var. *silvestre* and *C. endivia* var. *latifolium*) chicory were kindly provided by an Italian producer (Borgoricco, PD). Both vegetables have received the Protected Geographical Indication (PGI) status. For each salad, the analysis were performed 24h after the gathering of the vegetables (samples indicated as TT1 for "Treviso tardivo" and VC1 for "Variegato di Castelfranco"); 24h after gathering, cutting and washing of the vegetables (TT2 and VC2); 48h after the gathering, cutting, washing, packaging in boxes and storage at 4°C (TT3 and VC3); 8 days after the gathering and storage at 4°C (TT4 and VC4); and finally 8 days after the gathering, cutting, washing, packaging in boxes and storage at 4°C (TT5 and VC5). Three different pools of sample were tested in duplicate.

2.3 Sample preparation

The extraction of the water soluble components (namely juice) were performed as follow: all samples were homogenized, centrifuged at 5000 rpm for 4 min to completely separate the juice from the solid parts, filtered through a paper filter (Carlo Erba, diameter=150mm), and then through Millipore membranes of cellulose acetate/cellulose nitrate mixed esters (0.45μm).

2.4 Anti-Peroxyl Radical Activity (AA %)

The anti-peroxyl radical activity, based on coupled oxidation of linoleic acid and β-carotene, was determined according the method of Taga, slightly modified [11, 12]. Briefly, a 1.5ml aliquot of β-carotene/chloroform solution (1 mg/ml) was added to a conical flask together with 20mg of linoleic acid and 200mg of Tween 20. Then, the chloroform was evaporated (under reduced pressure at temperature lower than 30°C). Millipore grade water (50μl) was added to the dried mixture, and the mixture was shaken. Two aliquots (200μl) of each sample were added to 2.5ml of β-carotene emulsion in test tubes. The absorbance of the first aliquot was immediately measured at 470nm, while the absorbance of the second aliquot was measured after 30 min of incubation in a water bath at 50°C. Each sample was compared with the blank (emulsion without β-carotene). To avoid any interfering effect of juice color in the calculation of the β-carotene degradation rate, an aliquot of each juice solution was added to 2.5ml of blank. Controls were also prepared using 200μl of Millipore grade water added to 2.5ml of β-carotene emulsion and treated as the corresponding sample.

These mixtures were read spectrophotometrically, and the measured absorbance was subtracted from that of the corresponding sam-

ple. The degradation rate of β-carotene in the samples and in the controls was calculated using the equation:

$$\text{Degradation rate} = \ln(A_0/A_t)/t$$

where, A_0 is the difference of absorbance of the sample minus the absorbance of the blank at time 0, A_t is the difference of absorbance of the sample minus the absorbance of the blank at time t, t is 30 min of incubation in a water bath at 50°C.

AA was expressed as the percent of inhibition in the sample relative to the corresponding control. A Trolox C (Sigma) solution was also assayed for AA at three different concentrations (50μM AA%=65.23±1.43; 100μM AA%= 82.78±1.65; 200μM AA% = 85.06±0.85). Values are means of three independent experiments performed in duplicate.

2.5 DPPH Assay (Antiradical Activity, ARA%)

A 50μl aliquot of juices (sample) or a 50μl aliquot of KH_2PO_4/NaOH buffer (pH=7.4) (control sample) was added to 1950μl of a 6×10^5 mol/l methanol/KH_2PO_4/NaOH buffer (50:50 v/v) DPPH• solution. The decrease in absorbance was determined at 515nm when the reaction had a steady state (after 20 min of reaction).

The percent scavenger activity (ARA %) against DPPH was expressed as the percent of inhibition relative to the control. The scavenger activity was also determined for a methanol solution of Trolox C (Sigma), which was assayed at three different concentrations (100μM ARA%=46.54±0.54; 200μM ARA% = 70.99 ± 1.09; 250μM ARA % = 74.11±0.99) [11-15].

The values are the mean of the results of three independent experiments each performed in duplicate.

2.6 Metal Chelating Ability (MCA)

The percent chelating activity of vegetable juices on Fe^{2+} was measured according to the method described previously by Wang et al [16, 17] with some modification, by adding 50μl of $FeCl_2\cdot4H_2O$ (2.0mM) to 20μl of tenfold diluted extracts (sample) or to 20μl of methanol (control sample). The reaction started with the addition of 200μl of ferrozine (5mM) and then 1230μl of methanol were added to prepare the final volume of 1500μl. After 10 min of incubation, the reaction mixture was read at 562nm against the blank. EDTA was used as positive control and the metal chelating ability was calculated in accordance with the following equation:

$$\text{MCA}\ (\%) = [(A_{cs}-A_s)/A_{cs}] \times 100$$

Where A_{cs} is the absorbance of control sample and A_s is the absorbance of the tested samples. The data are the mean values of the results of three independent experiments each performed in duplicate.

2.7 Reducing Power Assay (RP)

The reducing power was determined as previously described [18,19], with some modifications. 1ml aliquot of vegetable juice (diluted 1:500 μ) was mixed in a test tube with 0.4ml of potassium hexacyanoferrate (III) (0.02M), 0.05ml of hydrochloric acid (0.01M), 0.4ml of iron (III) chloride (0.02M), and 0.7ml of distilled water. After 15 min of incubation in darkness, the absorbance was read at 720nm. The reducing substances were expressed as the concentration (μM) of ascorbic acid solution, used as standard, showing the same reducing power. Values are means of three independent experiments performed in duplicate.

2.8 Statistical analysis

Each set of results was first studied using a one-way ANOVA to ascertain the effect of the applied treatments on the values of the assayed markers.

A paired t-test of the means was then used to verify the possible differences in the mean results of each marker on the samples.

Data processing and figures were made using Microsoft Excel 2007 and R version 3.0.1 (2013-05-16) Copyright (C) 2013 (The R Foundation for Statistical Computing).

3. Results and Discussion

The results of antioxidant/antiradical activity obtained in all tests for the juices submitted to the different treatments are reported in Table 1. All samples showed good anti-peroxyl and anti-DPPH radical activities with values generally higher than that shown by a 50μM and a 100μM Trolox C solutions, respectively. Conversely, the juices possessed only a weak metal chelating property with “Treviso Tardivo” values always higher than the corresponding “Variegato di Castelfranco” ones. Finally, the reducing power of different juice solutions decreased with the increasing of storage time for both vegetables, and it is presumed that it was related to the vegetable leaves presented in treatments.

Table 1. Antioxidant and antiradical experimental data collected for "Treviso tardivo" (TT) and "Variegato di Castelfranco" (VC) salads in the applied tests.

Sample	Anti-peroxyl radical activity (%)	Antiradical activity (%)	Metal chelating ability (%)	Reducing power (□M)[e]
TT1	72.63 ± 5.63	69.35 ± 2.83	25.11 ± 1.38	79.8 ± 2.0
TT2	69.76 ± 5.58	67.63 ± 1.15	24.11 ± 0.36	65.8 ± 1.4
TT3	61.47 ± 4.85	65.80 ± 0.81	22.57 ± 0.36	60.8 ± 0.2
TT4	65.93 ± 1.04	63.76 ± 2.04	20.42 ± 0.57	44.2 ± 2.0
TT5	52.93 ± 1.04	63.43 ± 1.71	19.97 ± 0.48	38.6 ± 1.4
VC1	77.14 ± 4.36	73.48 ± 3.39	16.00 ± 0.40	68.6 ± 0.9
VC2	70.70 ± 6.70	68.26 ± 2.48	15.22 ± 0.33	57.6 ± 0.4
VC3	64.38 ± 3.91	68.81 ± 0.96	14.64 ± 0.29	54.2 ± 0.4
VC4	61.08 ± 1.96	63.56 ± 2.80	14.05 ± 0.21	39.6 ± 0.5
VC5	60.79 ± 3.49	64.60 ± 0.95	14.03 ± 0.54	33.8 ± 0.7
Standard Reference	65.23 ± 1.43[a]			
	82.78 ± 1.65[b]	46.54 ± 0.54[b]		
	85.06 ± 0.85[c]	70.99 ± 1.09[c]		
		74.11 ± 0.99[d]		

[a] Trolox C 50 μM; [b] Trolox C 100 μM; [c] Trolox C 200 μM; [d] Trolox C 250 μM; [e] ascorbic acid solutions (μM: micromolar)

For the meaning of sample numbering see the Materials and Methods section

The statistical analysis demonstrated that all treatments had a remarkable effect on the investigated properties with a high level of statistical significance ($p<0.001$).

In Table 1, two groups of samples could be selected, based on the treatment applied to the vegetables. The first group included only the harvested and stored samples (samples TT1 and VC1 and samples TT4 and VC4, n=12 per vegetable), and the second one the manipulated samples including cut, washed, and packaged in boxes in addition to harvesting and storage (samples TT and VC numbered 2, 3, and 5, respectively, n=18 per vegetable).

In general, all the investigated properties showed a marked decrease starting from the vegetables analyzed 24h after harvesting (TT1 and VC1 samples) to the vegetables immediately stored at 4°C after harvesting and analyzed after 8 days (TT4 and VC4). The differences in the mean values of TT1 and VC1, and TT4 and VC4 were highly significant in all cases ($p<0.001$) except for "Treviso Tardivo" anti-peroxyl radical activity ($p<0.05$), but this could be explained considering the high variance of the data obtained by this assay.

Going more into details, anti-peroxyl radical activity was higher in fresh samples as compared to 8- days stored samples (activity of sample 1>sample 4, and samples 2 and 3>sample 5); however, the difference between 24h and 48h (short-term period) AA% values was statistically significant only in the case of the VC type salad (sample 2> sample 3, $p<0.01$).

$DPPH^{\bullet}$ antiradical activity showed a remarkable decrease over time when no treatment was applied (ARA% sample 1> sample 4, $p<0.001$ for both type of salads). As regards the manipulated vegetables (samples 2, 3, and 5), the ARA% values for TT salad showed a clear downward trend, while for VC salad a marked decrease in ARA% was detectable only when the storage time was longer than 2 days (samples 2 and 3 were not statistically different, while both were different from sample 5, $p<0.01$).

Metal chelating ability decreased over time and resulted to be influenced by the applied treatments in both types of salads with a high degree of statistical significance ($p<0.001$). Overall, the decrease in this property followed the trend sample 1>2>3>4>5 in both salads, but the MCA% values for TT salad were always much higher than the corresponding values for VC salad (see also Figure 1).

Reducing power significantly decreased with time in the two types of salads. Manipulation of the samples also remarkably contributed to the decrease of this property (Figure 2).

The tested chicory salads possessed antioxidant and radical scavenging properties both in *in vitro* and *ex vivo* systems, as previously reported in our studies [11,12]. In this work, we applied four different assays for evaluating the effects of the different treatments applied to ready-to-eat "Treviso Tardivo" and "Variegato di Castelfranco" salads on such properties.

All the tests were suitable for our purpose. In fact, the sensitivity (expressed by the ability to demonstrate a change in the tested properties following the applied treatments) and the precision (expressed by the scatter of the data in replicate analysis, measured by the RSD %) of the methods were satisfactory.

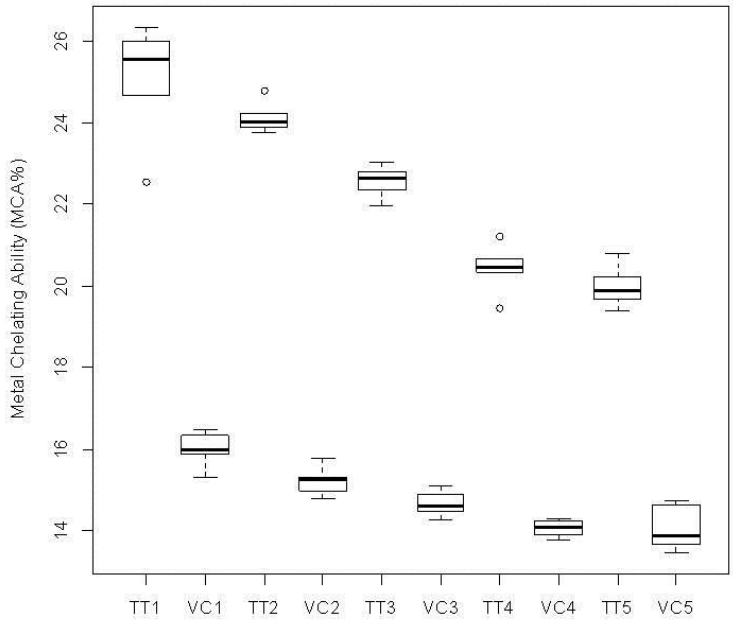

Figure 1. Box-and-whisker plot of the Metal Chelating Ability (MCA%) results for "Treviso tardivo" (TT) and "Variegato di Castelfranco" (VC) salads.

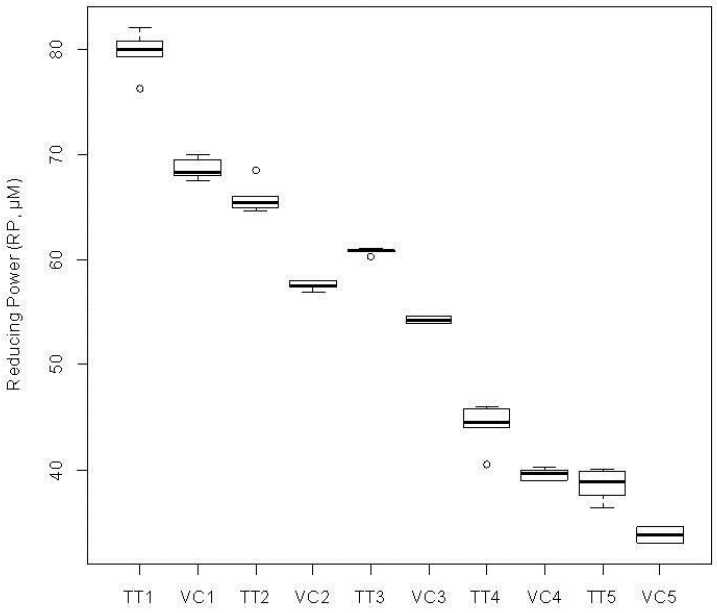

Figure 2. Box-and-whisker plot of the Reducing Power (RP) assay results for "Treviso tardivo" (TT) and "Variegato di Castelfranco" (VC) salads.

Overall, the ARA, MCA, and RP assays showed better precision (average RSD≤ 3% and max RSD≤6%) than AA assay (average RSD≤6% and max RSD≤9%) for both types of salads.

The low decrease in all the antioxidant/antiradical activities registered for both chicories could be due to the higher exposure of the water soluble antioxidant compounds to atmospheric oxygen due to the cutting process to which the vegetable underwent or to the loss of a part of these compounds during the washing procedure after cutting.

In fact, the vegetables contain different completely water soluble or partially water soluble components including phenolic acid der-

ivatives and highly glycosylated flavonoids with antioxidant activity as demonstrated in our previous investigations (10, 12, 20). A further decrease in the antioxidant properties was observed for the juices obtained from the minimally treated vegetables stored at 4°C by the increasing of storage period, but the values were constant in the juices from the whole vegetable leaves after 8 days storage in the same conditions. This could be due to the generation of oxidized compounds as a consequence of enzymatic activity during storage (21).

4. Conclusion

In conclusion, the results indicated that the minimal processes applied for the ready-to-eat vegetable preparation induced negative effects on the antioxidant/antiradical properties measured by the four chemical assays. The decrease was more remarkable after 48h of vegetable gathering and after 8 days of storage at 4°C. It was also highly significant when compared with the corresponding untreated samples analyzed at the same time of storage. The research is going on in order to evaluate the effect of these treatments on the qualitative and quantitative compositions of the polyphenolic fraction including compounds with different polarity.

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