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Antioxidant Activity of Minimally Processed Red Chicory (*Cichorium intybus* L.) Evaluated in Xanthine Oxidase-, Myeloperoxidase-, and Diaphorase-Catalyzed Reactions

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Minimally processed red chicory products (*Cichorium intybus* L. var. *silvestre*) were studied for their polyphenol content and antioxidant activity evaluated by using the synthetic 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl radical and three model reactions catalyzed by relevant enzymatic sources of reactive oxygen species, namely, xanthine oxidase, myeloperoxidase, and diaphorase. Products were analyzed at the time of production and after storage at 4 °C within either a gas permeable film or a gas barrier film. The antioxidant activity and contents of hydroxycinnamic acids and flavonoids decreased by less than 20% during storage of the minimally processed red chicory products. Total phenolics were significantly correlated with the antioxidant activity evaluated with both the synthetic radical and the enzyme-catalyzed reactions. On a molar basis, red chicory phenolics were as efficient as the reference compound Trolox in scavenging the synthetic radical. However, red chicory phenolics had a much higher inhibitory activity than Trolox in the model enzymatic systems.

KEYWORDS: ***Cichorium intybus* L.; antioxidant activity; xanthine oxidase; myeloperoxidase; diaphorase; 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl**

INTRODUCTION

Cichorium intybus L. has been regarded as a food source of nutritionally relevant phytochemicals since antiquity (*1*). The potential health benefit of *C. intybus* has been related to its phenolic compounds. In fact, plant polyphenols are recognized as phytochemicals contributing to disease protection. The bioavailability and bioefficacy of polyphenols in humans have been recently reviewed (*2*). Polyphenols are believed to have a role in vivo in inhibiting reactive oxygen species (ROS)-mediated reactions, which have been linked with the initiation and progression of a number of pathological processes (*3, 4*). The red variety of *C. intybus* has been found to possess the highest polyphenol content of a selection of leafy vegetables that are consumed fresh, namely, lettuce (*Lactuca sativa* L.), endive (*Cichorium endivia* L.), rocket (*Eruca sativa* Miller), wild rocket (*Diplotaxis tenuifolia* L.), and Buck's horn plantain (*Plantago coronopus* L.) (*5, 6*).

Leafy vegetables are generally processed by washing, cutting and packaging, and cold storage (minimal processing). Plants have developed adaptation mechanisms to cope with stressful conditions, such as those caused by minimal processing. One of the responses to the wounding caused by processing in lettuce and endive varieties is the activation of phenylalanine ammonia lyase followed by the synthesis of phenylpropanoids. These compounds can be used as defense mechanisms or may be oxidized by polyphenol oxidase, peroxidase, and phenolase (*7, 8*). Therefore, the level of phenolics in wounded tissues is the result of a balance between their synthesis rate and their utilization rate. The phenolic contents of leafy vegetables account for their antioxidant activity toward the peroxyl radicals generated by lipid peroxidation (*6*), for the scavenging of the synthetic 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) radical, and for the Fe-chelating activity (*9*).

Besides autocatalytic lipid peroxidation and metal-catalyzed reactions, ROS generation in vivo involves some enzyme-mediated reactions (*4*). Xanthine oxidase (XOD) is one of the main enzymatic sources of ROS in vivo. XOD is involved in the oxidative damage that occurs after reperfusion of ischemic tissues, in brain edema and injury, and in vascular permeability changes. In fact, XOD is present in several types of cells as a dehydrogenase enzyme that oxidizes xanthine or hypoxanthine to uric acid. Under certain conditions, the dehydrogenase is converted to an oxidase enzyme. Upon this conversion, the enzyme reacts with the same electron donors, but in the reoxidizing steps, it reduces oxygen instead of NAD^+, thus producing superoxide or hydrogen peroxide (*10*).

Another source of strong oxidants in vivo is myeloperoxidase (MPO), which has a role in inflammatory diseases. MPO is a neutrophil enzyme that catalyzes oxidation of chloride ions by hydrogen peroxide, resulting in hypochlorite production. The

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cytotoxicity of this reaction contributes to the killing of bacteria in the host defense system. However, hypochlorite generated by MPO might also inactivate R1-antiproteinase, contributing to tissue damage (*11*).

Enzymatic systems involved in xenobiotic detoxification may also indirectly contribute to ROS production. Among these enzymes, diaphorase (DIA, [NADPH:quinone oxidoreductase]) is believed to be involved in the toxicity of quinoid compounds (*12*). It is another flavoenzyme present in most animal tissues and involved in a number of electron-transfer processes. It also catalyzes the two-electron reduction of quinones to hydroquinones, allowing their disposal from the cells after conjugation of hydroquinones with glucuronic acid or sulfate. However, the hydroquinones formed by DIA can autoxidize, with the transfer of two electrons to molecular oxygen and the formation of two superoxide radicals plus a quinone. The quinone, in turn, can be reduced again by DIA, thereby closing a cycle of enzyme reduction/oxidation yielding ROS.

There are no available data in the literature on the antioxidant activity of red chicory products evaluated by enzyme-mediated ROS generation, despite the potential effectiveness of this crop due to its high antioxidant content. The aim of the present study was to assess the phenolic content and the antioxidant activity of minimally processed red chicory by model systems involving XOD, MPO, and DIA. For comparison, the radical scavenging activity on the DPPH radical was also evaluated. The study was carried out on minimally processed red chicory products at the time of production and after wounding due to storage either in a gas-permeable film or in a film with gas barrier properties for 6 days at 4 °C (the use by date).

MATERIALS AND METHODS

Chemicals. Cyanidin 3-*O*-glucoside, chicoric acid, chlorogenic acid, quercetin, quercetin 3-*O*-glucoside, and quercetin 3-*O*-rutinoside (rutin) were purchased from Extrasynthese (Lyon, France). Luteolin, β-glucuronidase (type b-10 from bovine liver), α-keto-γ-methiolbutyric acid (KMB), 1-aminocyclopropane-1-carboxylic acid (ACC), xanthine, 5-hydroxy-1,4-naphthoquinone (juglone), DPPH, and MPO (from human leukocytes) were purchased from Sigma-Aldrich (Milano, Italy). XOD (from cow's milk) and DIA (from pig heart) were purchased from Roche (Monza, Italy).

Red Chicory Samples. Minimally processed vegetables were grown near Chioggia (Italy) and processed by an industrial producer of ready-to-eat salads. Samples 1, 2, and 3 were from three independent lots produced on different days under the same processing conditions. Each was composed of 250 g of mixed salad (*C. intybus* var. *silvestre* "Chioggia", *C. endive*, *E. sativa*, and *Daucus carota*) packed in semirigid polypropylene trays (22 cm × 15 cm) and overwrapped with a 13 μm thick polyvinyl chloride (PVC) film. The high oxygen and carbon dioxide permeability and good water barrier properties of 13 μm thick PVC allowed minimization of passive atmosphere modification and water loss, respectively (*13*). Therefore, these packaging conditions allowed storage of samples in air. Samples 4, 5, and 6 were three independent lots produced on different days under the same processing conditions. Each was composed of 250 g of mixed chicories (*C. intybus* var. *silvestre* "Chioggia" and *C. endive*) packed in heat-sealed bags (25 cm × 29 cm) made of a 35 μm thick monooriented polypropylene (OPP) film. The gas transmission rates for O_2 and CO_2 of the 35 μm thick OPP film were 5500 and 10000 mL/m^2 day atm, respectively, at 23 °C.

Sample 7 was an independent lot produced under the same processing conditions as samples 4−6. Half of the packages from this lot were opened at the time of production and packed in semirigid polypropylene trays (22 cm × 15 cm) and overwrapped with a 13 μm thick PVC film before storage.

Minimally processed vegetables in their packages were stored in a thermostatic chamber for 6 days (i.e., until the use by date) at 4 °C in the dark. Samples 1−6 were analyzed at the beginning and end of the storage time. Sample 7 was analyzed at the beginning of the storage time and after 1, 2, 3, 4, and 6 days from packaging. At each sampling time, four packages of each lot were tested.

O_2 and CO_2 concentrations were assayed by a CheckMate II analyzer (PBI Dansensor, Segrate, Milano, Italy). At the end of the storage time, O_2 and CO_2 concentrations inside the PVC packages were the same as those of air. O_2 and CO_2 concentrations were 4 ± 1 and 10 ± 2%, respectively, in the OPP packages.

Sample Extraction. For each package of minimally processed vegetables, only red chicory (*C. intybus* var. *silvestre* "Chioggia") was analyzed. Red chicory (50 g) was added to 500 mL of methanol containing 4% formic acid. The mixture was stirred gently under nitrogen for 2 h at room temperature. An aliquot of 500 mL of water containing 4% formic acid was then added to the mixture, which was once again stirred gently under nitrogen at room temperature for 2 h. This solvent was chosen since 50% methanol containing 1% formic acid was found to be a better solvent for lettuce phenolics than those containing lower or higher methanol percentages (*14*). In the present study, the formic acid content was increased to 4% to stabilize anthocyanins. The extract was centrifuged (12000*g* at 5 °C for 10 min) and filtered through Whatman #4 filter paper.

For the evaluation of antioxidant activity with the enzymatic systems, the chicory extracts in 50% aqueous methanol containing 4% formic acid (4 mL) were dried under vacuum and dissolved in 0.2 M phosphate buffer, pH 7.4 (4 mL). To separate the MW > 10 kDa components from the MW <10 kDa components, an aliquot (2 mL) of the chicory extract dissolved in 0.2 M phosphate buffer, pH 7.4, was ultrafiltered by a membrane with a 10 kDa cutoff (Millipore, Milano, Italy), to recover 0.5 mL of retentate. To the retentate, 2 mL of 0.2 M phosphate buffer, pH 7.4, was added, and the procedure was repeated four times.

Determination of Phenolic Compounds. The chicory extracts in 50% aqueous methanol containing 4% formic acid were diluted with 4% formic acid in water (1:2.5), and phenolics were identified using a model 600 high-performance liquid chromatography (HPLC) pump coupled with a model 2996 photodiode array detector, operated by Empower software (Waters, Vimodrone, Italy). A 250 mm × 4.6 mm i.d, 5 μm Symmetry reverse phase C-18 column (Waters, Vimodrone, Italy) equipped with a Symmetry C-18 precolumn was used. The chromatographic separation was carried out according to a procedure reported previously (*15*). Formic acid (5%) was added to both methanol and water before preparing the following mobile phase: water/methanol (95:5, v/v) (A), water/methanol (88:12, v/v) (B), water/methanol (20:80, v/v) (C), and methanol (D). The following gradient elution was used: 0−5 min, 100% A; 5−10 min linear gradient to reach 100% B; 10−13 min, 100% B; 13−35 min linear gradient to reach 75% B and 25% C; 35−50 min linear gradient to reach 50% B and 50% C; 50−52 min linear gradient to reach 100% C; 52−57 min, 100% C; and 57−60 min, 100% D. The injection volume was 20 μL. The flow rate was 1 mL/min.

Standards of chlorogenic acid, chicoric acid, quercetin, luteolin, quercetin 3-*O*-glucoside, and cyanidin 3-*O*-glucoside were used to identify peaks by retention times and UV−vis spectra. Other hydroxycinnamic acids were detected by UV−vis spectra (*15*). Luteolin and quercetin conjugates were identified by their UV−vis spectra (*16, 17*) and by acid hydrolysis (*8*). Among luteolin and quercetin conjugates, the glucuronides were identified after enzymatic hydrolysis with β-glucuronidase. Deglucuronidation was carried out according to a procedure reported previously (*8*). The chicory extracts in 50% aqueous methanol containing 4% formic acid (0.5 mL) were dried under nitrogen atmosphere and redissolved in 0.5 mL of 0.5 M sodium acetate buffer, pH 5.0, and then, 20 μL of 400 U/mL of β-glucuronidase was added. The mixture was incubated at 37 °C for 30 min, and 0.75 mL of methanol containing 4% formic acid was added prior to HPLC analysis. The acylated derivatives of cyanidin 3-*O*-glucoside and quercetin 3-*O*-glucoside were identified by UV−vis spectra and by alkaline hydrolysis (*18*).

The phenolic compounds were quantified by calibration curves built with external standards. The anthocyanins were quantified as cyanidin

3-*O*-glucoside at 510 nm, hydroxycinnamic acids as chlorogenic acid at 330 nm, and flavonols and flavones as rutin at 350 nm. Concentrations were expressed as mg per kg of fresh product.

Antioxidant Activity. *XOD/Xanthine System.* This system contained 0.2 M phosphate buffer, pH 7.4; 0.5 mM xanthine (in 10 mM NaOH); 0.08 units of XOD; 1.25 mM KMB; and various aliquots of chicory extract dissolved in 0.2 M phosphate buffer, pH 7.4. Both the effects of whole chicory extract and the effects of the MW > 10 kDa components on the XOD-catalyzed reaction were separately analyzed. The reaction was carried out at 37 °C for 30 min, followed by gas chromatographic determination of the ethylene released from KMB (*19*).

MPO/NaCl/H_2O_2 System. This system contained 0.2 M phosphate buffer, pH 6.0; 150 mM NaCl; 0.1 mM H_2O_2; 0.025 units of MPO; 1.25 mM ACC; and various aliquots of chicory extract dissolved in 0.2 M phosphate buffer, pH 7.4. The reaction was carried out at 37 °C for 30 min, followed by measurement of the ethylene released from ACC (*19*).

DIA/NADH/Juglone System. This system contained 0.2 M phosphate buffer, pH 7.4; 0.1 mM NADH; 0.05 mM juglone; 0.75 units of DIA; 1.25 mM KMB; and various aliquots of chicory extract dissolved in 0.2 M phosphate buffer, pH 7.4. The reaction was carried out at 37 °C for 30 min, followed by gas chromatographic determination of the ethylene released from KMB (*20*).

Ethylene. Ethylene was determined by GC equipment consisting of a Varian Aerograph 3300 with a Varian integrator and a 100 cm × 1/8 in. i.d. deactivated aluminum oxide column. The column temperature was 60 °C, the injection temperature was 80 °C, and the FID temperature was 225 °C.

DPPH Scavenging Test. This assay was performed as described previously (*20*). Briefly, different dilutions of the red chicory extracts in methanol:water (50:50) containing 4% formic acid (1 mL) were added to a 25 mg/L methanolic solution of DPPH (2 mL). The decrease in absorbance was determined at 515 nm after 30 min (when a constant value was reached) by a Jasco UVDEC-610 spectrophotometer (Jasco Europe, Cremella, LC, Italy).

Antioxidant Activity Quantification. In each enzymatic system, control reactions were prepared with no addition of chicory extract. The percentage of inhibition of the control reaction rate in the presence of chicory extract was calculated, and a dose−response curve was constructed. The amount of chicory required to inhibit the reaction by 50%, I_{50}, was interpolated by the dose−response curve.

In the DPPH test, the percent decrease of DPPH concentration was calculated with respect to the initial value, after 30 min of reaction. A dose−response curve was constructed, and the amount of chicory required to lower the initial DPPH concentration by 50%, I_{50}, was interpolated.

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma, Milano, Italy) was used as a reference antioxidant in all enzymatic reactions and in the DPPH test. The antioxidant activity of chicory was expressed as Trolox equivalents. Trolox equivalents are the ratio of the I_{50} of Trolox (nmol) to the I_{50} of the sample (mg, fresh weight).

Statistical Analysis. Linear regression of data and analysis of variance were conducted with Statgraphics 5.1 (STCC Inc., Rockville, MD); Fisher's least significant difference (LSD) procedure ($p < 0.05$ or $p < 0.1$) was used to discriminate among the means.

RESULTS AND DISCUSSION

As the initial part of this study, the changes in the DPPH radical scavenging activity of a minimally processed red chicory product, packed in either a PVC or an OPP film, were evaluated over 6 days of storage. These films are widely used for packaging of minimally processed vegetables, particularly for red chicory products. PVC is a gas permeable film, whereas OPP is characterized by O_2 and CO_2 barrier properties and allows the generation of a modified atmosphere within the package (*13*). As shown in **Table 1**, the radical scavenging activity of minimally processed red chicory progressively decreased during storage in a PVC film, whereas it decreased on the first day of storage in an OPP film and then increased. In both cases, wounding due to storage of the products up to the use by date caused a slow variation in the DPPH radical scavenging activity with respect to the initial value. Therefore, the study was then carried out on red chicory products analyzed at the moment of production and at the end of their commercial lives to investigate their polyphenol content and antioxidant activity.

Table 1. DPPH Scavenging Activity (mmol Trolox Equivalents/kg Fresh Product) of a Minimally Processed Red Chicory Product during 6 Days of Storage in either a PVC Film or an OPP Film at 4 °C[a]

	DPPH scavenging activity	
storage time	PVC packaging	OPP packaging
initial	5.7 d ± 0.05	5.7 b ± 0.05
1 day	5.6 d ± 0.08	5.3 a ± 0.03
2 days	5.3 c ± 0.11	5.8 b ± 0.10
3 days	5.3 c ± 0.11	6.1 c ± 0.06
4 days	5.1 b ± 0.09	6.1 c ± 0.12
6 days	4.9 a ± 0.06	6.3 d ± 0.13

[a] Results represent the mean ± SD of four packages. Different letters within a column (a−d) indicate significant differences with respect to storage time (LSD, $p < 0.05$).

Polyphenol Content. The HPLC analysis of the red chicory var. *silvestre* "Chioggia" extracts revealed the presence of chlorogenic acid (5-*O*-caffeoylquinic acid, **2**), chicoric acid (dicaffeoyltartaric acid, **5**), unidentified caffeic acid derivatives (**1**, **6**, and **10**), cyanidin-3-*O*-glucoside (**3**), cyanidin 3-*O*-malonyl glucoside (**4**), a luteolin glucuronide, tentatively identified as luteolin 7-*O*-glucuronide (**7**), quercetin 3-*O*-glucoside (**9**), and two other quercetin conjugates, tentatively identified as quercetin 3-*O*-glucuronide (**8**) and an acylated derivative of quercetin 3-*O*-glucoside (**11**) (**Figures 1** and **2**). The amounts of **2**−**5** observed in this study in minimally processed products (**Table 2**) were comprised within the concentration range previously found in four fresh samples of the same red chicory variety (*5, 6*). Compounds **7**−**9** were found in red chicory (*5, 21*). An acylated derivative of **9** was identified in some lettuce and endive varieties (*8, 22*), but it has never been found in red chicory until now.

At the time of production, there were major differences in the phenolic content in the red chicory samples, all belonging to the var. *silvestre* "Chioggia". This result is in agreement with the highly variable level observed in other varieties of leafy vegetables (*8*). In these red chicory samples, variations within variety are likely due to different growing conditions, in particular different exposure to UV radiation and climate, since this red chicory is cultivated in open fields.

The changes in phenolic contents of minimally processed red chicory products after 6 days of storage in either a PVC or an OPP film are reported in **Table 3**. In the samples stored in a PVC film, total hydroxycinnamic acid and total flavonol and flavone contents decreased by 17% and total anthocyanin content decreased by 19%. Storage in an OPP film did not cause significant changes in the phenolic content. In agreement with this latter result, it was reported that elevated CO_2 concentrations inhibit polyphenol oxidase activity. Specific CO_2 concentrations at which this effect can be observed depend upon the commodity, cultivar, temperature, duration of storage, and interaction between O_2 and CO_2 (*13*). However, the concentration of CO_2 and O_2 that affects phenolic metabolism in red chicory is unknown, and this aspect was beyond the aim of this study. From this portion of the study, the main result indicated that whatever the initial level and packaging conditions, the phenolic

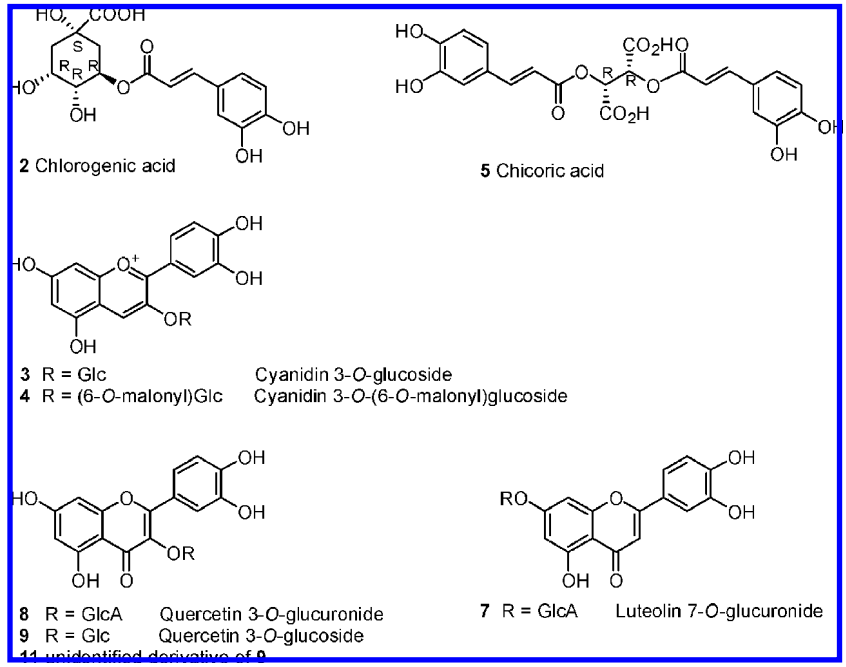

Figure 1. Main phenolic compounds reported in red chicory and discussed in this paper.

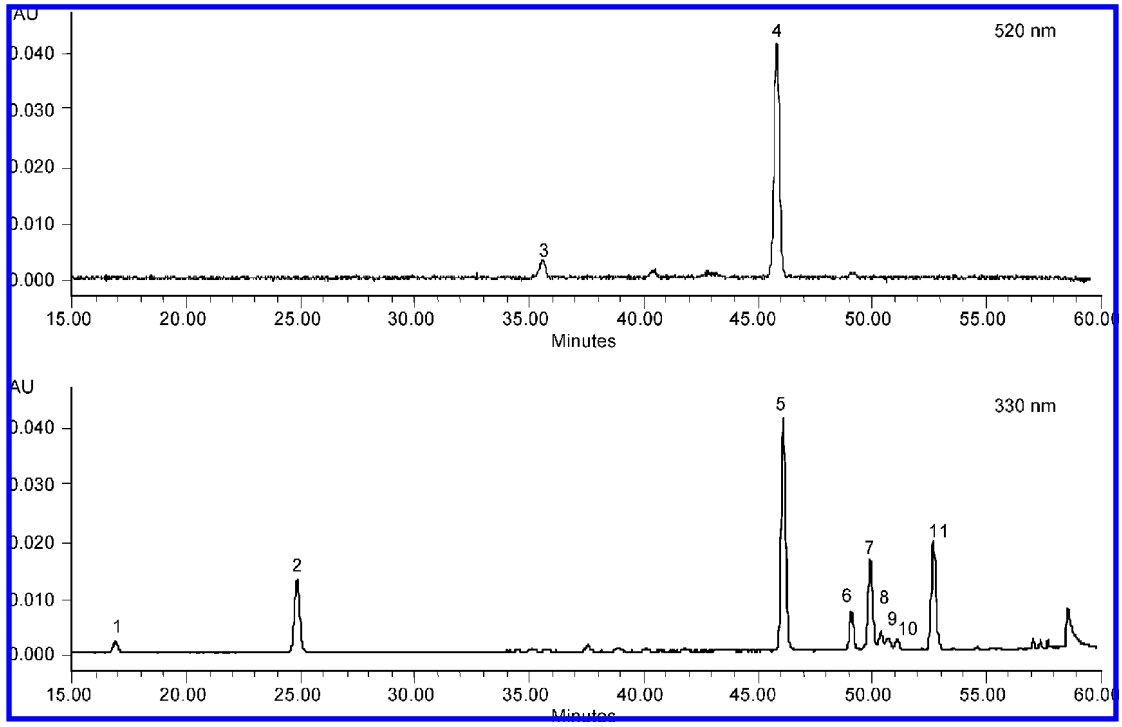

Figure 2. HPLC chromatograms of red chicory extract recorded at 330 and 520 nm: 1, unidentified caffeic acid derivative; 2, chlorogenic acid; 3, cyanidin 3-*O*-glucoside, 4, acylated derivative of 3, tentatively identified as cyanidin 3-*O*-(6-*O*-malonyl) glucoside; 5, chicoric acid; 6, unidentified caffeic acid derivative; 7, luteolin conjugate, tentatively identified as luteolin 7-*O*-glucuronide; 8, quercetin conjugate, tentatively identified as quercetin 3-*O*-glucuronide; 9, quercetin 3-*O*-glucoside; 10, unidentified caffeic acid derivative; and 11, quercetin conjugate, tentatively identified as an acylated derivative of quercetin 3-*O*-glucoside.

contents of all red chicory products after 6 days of storage at 4 °C (corresponding to the use by date) were, in general, higher than those found in other leafy vegetables and other food sources (*23*−*26*). In fact, total flavonol and flavone contents in red chicory were higher than 1000 mg/kg (**Table 3**). Total flavonol and flavone contents in the green varieties of lettuce and chicory are less than 445 mg/kg (*9, 23*). However, in a red lettuce variety, levels of flavonols and flavones up to 2846 and 380 mg/kg, respectively, have been observed (*23*). Regarding anthocyanins, the contents observed in red chicory were higher than 653 mg/kg (**Table 3**). In red lettuce varieties, the anthocyanin content is less than 456 mg/kg (*23*).

Red chicory, therefore, as observed for a few other leafy vegetables, could significantly contribute to the dietary intake of both flavonols, flavones, and anthocyanins. It is worth noting that, among different food sources, flavonoids are known to vary in the basic aglycone skeleton; the identity, number, and positions at which sugar are attached; the extent of sugar acylation; and the identity of the acylating agent. Therefore, a

Table 2. Phenolic Content (mg/kg Fresh Product) of Minimally Processed Red Chicory Products at the Time of Production[a]

	phenolic compounds							
	phenolic acids			flavone and flavonols		anthocyanins		
chicory	**2**	**5**	**1 + 6 + 10**	**7**	**8 + 9 + 11**	**3**	**4**	total
lot 1	543 b	842 b	265 c	645 c	320 a	53 a	527 a	3195 a
lot 2	388 a	822 b	396 d	604 a	998 c	56 ab	982 d	4261 c
lot 3	972 d	987 c	100 a	638 bc	522 b	139 d	648 c	4008 bc
lot 4	660 c	691 a	464 e	607 ab	366 a	55 ab	509 a	3354 a
lot 5	407 a	1015 c	205 b	1091 d	1567 d	63 b	1291 e	5366 d
lot 6	998 d	979 c	110 a	654 c	477 b	112 c	584 b	3915 b

[a] Phenolic acid contents were quantified as chlorogenic acid, flavone and flavonol contents were quantified as rutin, and anthocyanin contents were quantified as cyanidin 3-*O*-glucoside. Total phenolics are the summation of concentrations of individual compounds determined by HPLC. Different letters within a column (a−e) indicate significant differences among lots (LSD, $p < 0.05$). Results represent the mean of four packages. Coefficients of variation were always <10%.

Table 3. Variations in Phenolic Contents (mg/kg Fresh Product) of Minimally Processed Red Chicory Products after 6 Days of Storage in either a PVC Film or an OPP Film at 4 °C[a]

	phenolic compounds			
storage	C_{tot}	F_{tot}	A_{tot}	total
PVC packaging				
t_0	1777 ± 215	1242 ± 280	802 ± 197	3821 ± 476
t_6	1478 ± 203	1000 ± 153	653 ± 100	3131 ± 186
Δ	−17%*	−17%*	−19%*	−21%*
OPP packaging				
t_0	1844 ± 207	1496 ± 719	871 ± 362	4212 ± 933
t_6	1830 ± 192	1374 ± 661	981 ± 393	4190 ± 943
Δ	NS**	NS**	NS**	NS**

[a] C_{tot}, total phenolic acid contents; F_{tot}, total flavonol and flavone contents; and A_{tot}, total anthocyanin contents; the total phenolics are the summation of concentrations of individual compounds determined by HPLC. t_0 and t_6 are the phenolic contents at the beginning of storage and after 6 days, respectively. Results represent the means ± SD of three lots (for each of these lots, four packages were analyzed). Δ is the percent of losses after storage. NS, no significant variation after storage (*LSD, $p < 0.05$; **LSD, $p < 0.1$).

Table 4. Antioxidant Activity (mmol Trolox Equivalents/kg Fresh Product) of Minimally Processed Red Chicory Products at the Time of Production[a]

	antioxidant activity			
chicory	DPPH test	XOD/xanthine system	MPO/NaCl/H_2O_2 system	DIA/NADH/juglone system
lot 1	6.2 a	94 ab	173 b	196 a
lot 2	6.6 b	96 bc	180 b	330 d
lot 3	8.1 d	102 e	231 c	311 c
lot 4	7.0 c	92 a	148 a	215 b
lot 5	11.7 e	98 cd	220 c	357 e
lot 6	8.2 d	100 de	212 c	309 c

[a] Different letters within a column (a−e) indicate significant differences among chicory lots (LSD, $p < 0.05$). Results represent the means of four packages. Coefficients of variation were <3.5% for the DPPH test and the XOD/xanthine system, < 5% for the DIA/NADH/juglone system, and <10% for the MPO/NaCl/H_2O_2 system.

Table 5. Variations in the Antioxidant Activity (mmol Trolox Equivalents/kg Fresh Product) of Minimally Processed Red Chicory Products after 6 Days of Storage in Either a PVC Film or an OPP Film at 4 °C[a]

	antioxidant activity			
storage	DPPH test	XOD/xanthine system	MPO/NaCl/H_2O_2 system	DIA/NADH/juglone system
PVC packaging				
t_0	7.0 ± 0.9	97 ± 4	194 ± 30	278 ± 62
t_6	6.1 ± 0.5	87 ± 5	156 ± 36	238 ± 38
Δ	−13%*	−10%*	−20%*	−14%**
OPP packaging				
t_0	9.0 ± 2	97 ± 4	211 ± 34	293 ± 62
t_6	9.2 ± 2	97 ± 7	217 ± 24	291 ± 66
Δ	NS**	NS**	NS**	NS**

[a] t_0 and t_6 are the antioxidant activity values at the beginning of storage and after 6 days, respectively. Results represent the means ± SD of three lots (for each of these lots, four packages were analyzed). Δ is the percent of losses after storage. NS, no significant variation after storage (*LSD, $p < 0.05$; **LSD, $p < 0.1$).

better knowledge is required to compare the bioavailability and the potential health effects of flavonoids of different food sources.

The contents in caffeic acid derivatives in red chicory were higher than 1478 mg/kg (**Table 3**). Caffeic acid derivatives up to 2038 and 4363 mg/kg have been found in a red lettuce variety and a wild green chicory variety, respectively (*9, 23*). However, in general, in green lettuce and chicory varieties, the caffeic acid content is lower (*23*).

Antioxidant Activity. The changes in the antioxidant activity of minimally processed red chicory products were investigated in vitro. The antioxidant activity was first evaluated in terms of its ability to scavenge the DPPH radical. The DPPH radical scavenging activity of the red chicory at the time of production ranged from 6.2 to 11.7 mmol TE/kg (**Table 4**). This range is higher than that reported for the DPPH radical scavenging activity of some green lettuce and chicory products, which is 0.31−1.05 g TE/kg, corresponding to 1.2−4.2 mmol TE/kg (*22*). This is consistent with the higher polyphenol content of the red chicory var. *silvestre* "Chioggia". Accordingly, a previous study showed that the red variety of chicory had the highest DPPH scavenging ability of the various species of the genus *Cichorium* (*27*). However, a higher DPPH radical scavenging activity has been found in a red lettuce variety, which also has a remarkable polyphenol content (*23*).

The antioxidant activity was then evaluated by using three model systems involving XOD, DIA, and MPO as ROS generators. Minimally processed red chicory inhibited all of these reactions. The inhibitory effectiveness of red chicory products at the time of production ranged from 92 to 102 mmol TE/kg in the XOD/xanthine system, from 148 to 231 mmol TE/kg in the MPO/NaCl/H_2O_2 system, and from 196 to 357 mmol TE/kg in the DIA/NADH/juglone system (**Table 4**).

After storage of the samples in PVC, the antioxidant activity decreased by 13% in the DPPH test, by 10% in the XOD/xanthine system, by 20% in the MPO/NaCl/H_2O_2 system, and by 14% in the DIA/NADH/juglone system (**Table 5**). This behavior can be considered as representative of the effects of storage of minimally processed red chicory in air, whatever the vegetable mixture and the permeable packaging material used. In different minimally processed vegetables, antioxidant activity

changes ranged from a 51% decrease to an increase up to 44%. In fact, the effects of wounding on the antioxidant activity of vegetables are tissue-dependent (*28*). Red chicory was not included in the above-mentioned study; therefore, the results obtained in this study cannot be compared with those found previously.

Upon storage of minimally processed red chicory within an OPP film at 4 °C, the antioxidant activity remained unchanged, whatever the measuring system (**Table 5**). It is worth noting that this latter result was dependent on the specific nature of the barrier film and the vegetable mixture used and is not representative of other packaging conditions that generate different atmospheres. In fact, storage of minimally processed vegetables in a film with gas barrier properties causes a decrease in O_2 concentration and an increase in CO_2 concentration inside the package, depending on both the characteristics of each vegetable and the characteristics of the packaging film (*13*). Even though further investigations are needed to understand the effects of modified atmosphere on red chicory antioxidant activity, it can be observed that the packaging conditions currently applied by industry to store these products retained more than 80% of their antioxidant activity.

Correlation between Antioxidant Content and Antioxidant Activity. Plotting the antioxidant activity of red chicory products evaluated using both the enzyme-mediated reactions and the DPPH radical, as a function of the total phenolic content, resulted in linear relations ($p < 0.01$), with correlation coefficients of 0.94 for the DPPH test, 0.89 for the XOD/xanthine system, 0.80 for MPO/NaCl/H_2O_2 system, and 0.92 for the DIA/NADH/juglone system (**Figure 3**). The relations were the same for the fresh and stored chicory samples. The slopes of these plots correspond to the relative antioxidant activity. In the DPPH test, the relative antioxidant activity was 1.1 ± 0.1 mmol TE/mmol of red chicory phenolics ($p < 0.01$). This value indicates that red chicory phenolics were as efficient as the reference compound Trolox as DPPH scavengers. Accordingly, as compared to Trolox, purified standards of cyanidin 3-*O*-glucoside, quercetin 3-*O*-rutinoside, and chlorogenic acid have almost the same antioxidant activity in the DPPH test (*29*).

In the XOD/xanthine, MPO/NaCl/H_2O_2, and DIA/NADH/juglone systems, the relative antioxidant activities were 3.3 ± 0.5, 14 ± 3, and 33 ± 4 mmol TE/mmol of red chicory phenolics ($p < 0.01$). It is remarkable that, with respect to Trolox, red chicory phenolics showed similar antioxidant activity in the DPPH test and much higher antioxidant activity in the enzymatic systems, generating the superoxide radical plus hydrogen peroxide, hypochlorite, and quinonic radicals. This behavior cannot be attributed to the presence of residual superoxide dismutase and catalase activities in the red chicory extracts. In fact, the MW > 10 kDa components of the extract, which were separated from the MW < 10 kDa components by ultrafiltration, had no antioxidant activity in the XOD/xanthine system. Instead, the high inhibitory effect of chicory on the enzymatic reactions could be attributed to the phenolic compounds, which act as both potent ROS scavengers and enzyme inhibitors.

Regarding the XOD-catalyzed reaction, the high inhibitory activity of red chicory products found in this study is consistent with previous data obtained on pure phenolic standards. In fact, a study on the structure−activity relationship of flavonoids as inhibitors of XOD has revealed that the hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 are essential for a high inhibitory activity on XOD. Accordingly, quercetin and its glycoside rutin have been found to act as superoxide scavengers with additional XOD inhibitory activity (*30*). Chlorogenic acid has also been found to inhibit the XOD-catalyzed reaction by both mechanisms (*31*). Anthocyanins and anthocyanidins, including cyanidin and its derivatives, have a high inhibitory activity in the XOD-catalyzed reaction, which has been attributed entirely to the superoxide scavenging activity itself (*32*). Studies on phenolic ability to inhibit the MPO-catalyzed reaction have revealed that quercetin and its glycoside rutin also act as hypochlorite scavengers with additional MPO inhibitory activity (*33*). On the other hand, knowledge on the interaction of phenolic compounds with DIA is still to be investigated. Red chicory had greater inhibitory effects in the XOD- and MPO-mediated reactions than tomato products and greater inhibitory effects in the XOD- and DIA-mediated reactions than extra virgin olive oil, as we found previously (*34, 20*).

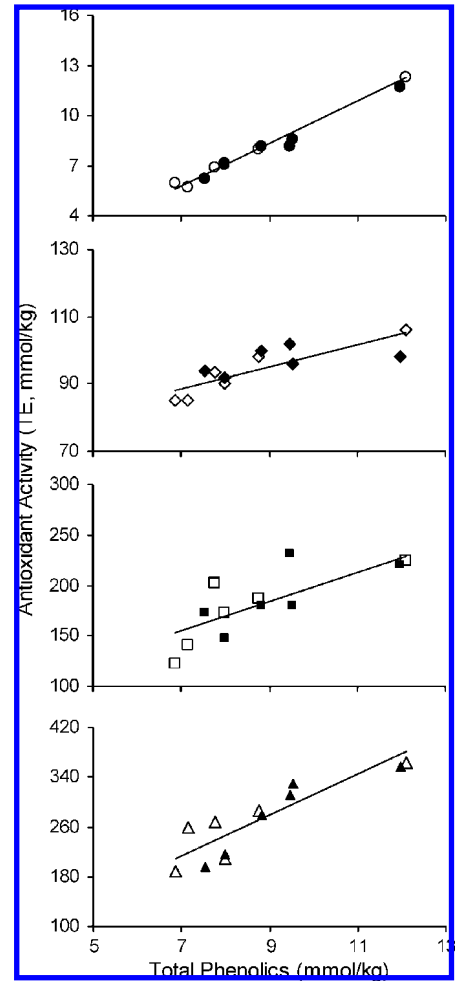

Figure 3. Correlation between the total phenolics (summation of concentrations of individual phenolics determined by HPLC in mmol per kg of red chicory) and the total antioxidant activity (in mmol of Trolox equivalents per kg of red chicory) measured by the DPPH (●, ○), the XOD/xantine (◆, ◇), the MPO/NaCl/H_2O_2 (■, □), and the DIA/NADH/juglone (▲, Δ) methods. Full symbols, samples at the time of production; open symbols, samples after storage for 6 days at 4 °C.

In conclusion, this study showed that all red chicory products, both at the time of production and after storage at 4 °C until the use by date, had a very strong antioxidant activity in the model enzymatic reactions generating the superoxide radical plus hydrogen peroxide, hypochlorite, and quinonic radicals. These products can provide additional advantages with respect to other food sources of antioxidants, since they contain high levels of phenolics that act both as radical scavengers and as inhibitors of ROS producing enzymes. All of the above-

mentioned ROS are produced in vivo during the initiation and progression of different pathological processes. Because chicory phenolics are absorbed by humans, it may be hypothesized that minimally processed red chicory could significantly contribute to the dietary intake of nutritionally relevant phytochemicals. The antioxidant activity of minimally processed red chicory was retained during storage. This latter information could also be useful for the utilization of red chicory byproducts as a source of bioactive components by the nutraceutical industry.

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