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DETERMINATION OF PHYTOCHEMICALS AND ANTIOXIDANT ACTIVITY IN BUTTERHEAD LETTUCE RELATED TO LEAF AGE AND POSITION

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ABSTRACT

The effect of leaf position on phytochemical distribution in butterhead lettuce was assessed by rings (from ring 1: outer leaves, to ring 6: inner leaves) or by zones (external, middle, internal). Maximum ascorbic acid was found in middle rings while chlorophyll and carotenoids gradually decreased from external to internal rings. Outermost leaves accumulate more phenolics and present more antioxidant activity as determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and trolox-equivalent antioxidant capacity methods. However, the presence of phenolics in this zone also contributes to the enzymatic browning. Quantitative but not qualitative differences were found in the phenolic profiles between inner and outer lettuce zones. The major phenolic compounds identified were the phenolic acids chicoric, chlorogenic and isochlorogenic. For all phenolic compounds, greater content was always found in the external zone except for caffeic acid. A high correlation between DPPH and total phenolics, chlorophyll and carotenes indicated that these compounds were major contributors of antioxidant activity in lettuce.

PRACTICAL APPLICATION

During lettuce development, each leaf had a different level of exposure to environmental conditions (light, humidity, nutrients absorption and temperature) and also inner leaves are younger than outer ones. These factors may affect the distribution of phytochemicals and antioxidant capacity in the lettuce head. Knowledge of the bioactive content and antioxidant capacity profile in lettuce plants could be of interest to consumers and the food industry for selecting the more suitable leaves to make salads or other ready-to-eat mixed vegetable dishes with high nutritional value. Additionally this study reveals, from a nutritional point of view, the losses value of regular greengrocers' practices that include the removal of the external lettuce leaves as storage advances and signs of senescence are evident.

INTRODUCTION

Lettuce (*Lactuca sativa* var. L) is one of the most popular leafy vegetables in the world preferably consumed fresh and in salad dishes. It is of particular interest in nutrition because of its content of antioxidants and phytochemicals including caffeic acid and its derivatives, flavonols, vitamins C and E, chlorophyll and carotenoids (Nicolle *et al.* 2004a; Llorach *et al.* 2008). These compounds are associated with health benefits such as a lower risk of developing cardiovascular disease, certain kinds of cancer and age-related degenerative processes (Hung *et al.* 2004; Soerjomataram *et al.* 2010; Wang *et al.* 2011). Several studies have shown the health effect of lettuce consumption in improving the lipid status and preventing tissue lipid peroxidation in rats and increasing plasma total antioxidant capacity and antioxidant levels in humans (Serafini *et al.* 2002; Nicolle *et al.* 2004b).

The lettuce head is an assemblage of leaves closely packed together over the growing point of the plant. Head formation results from the accumulation of young leaves under the layer of leaves covering the growing point (Wien 1997).

This pattern of growth constitutes an interesting model system that allows on the one hand to analyze the effect of the leaf position, as leaves are exposed to different environmental conditions before being harvested, such as light, humidity, nutrients absorption and temperature, and on the other hand, to establish the effect of leaf age, as inner leaves are younger than outer ones, both on the phytochemical composition of lettuce. In this sense, some lettuce quality indices are expressed by zones, such as external, middle and internal (Agüero *et al.* 2008), or by rings (Goñi *et al.* 2010) constituted by leaves with analogous characteristics.

Understanding the way in which phytochemicals are distributed in the whole lettuce plant could be of interest in the food industry to prepare healthier ready-to-eat salads using those leaves that present higher phytonutrient content and antioxidant activity. Additionally, it is a common practice among producers and retailers to remove the external lettuce leaves as time progresses because older leaves exposed to environmental conditions and handling present evident signs of deterioration. Knowledge of the phytochemical distribution in the lettuce head will reveal, from a nutritional point of view, the losses value of regular greengrocers' practices. Furthermore, consumers tend to eat from the middle part of the lettuce head toward the inner part as these parts look fresher, crispier and more tender without knowing the phytonutrient content of these zones (Ozgen and Sekerci 2011).

There are some studies that showed the distribution of phytochemicals in head lettuce (Hohl *et al.* 2001; Cano and Arnao 2005; Ozgen and Sekerci 2011). However, the data available mostly refer to measurements of particular antioxidants (Hohl *et al.* 2001) or closely related substances (Cano and Arnao 2005), which do not provide an overall approach to the phytochemical content of lettuce and do not give information of the total antioxidant activity of the vegetable. Additionally, those works that measure total antioxidant activity in lettuce have only used one or two methods of antioxidant capacity (Cano and Arnao 2005; Li *et al.* 2010; Ozgen and Sekerci 2011). It is known that the antioxidant properties of food matrices are due to the presence of a complex mixture of compounds with diverse structure that reacts differently to different radical or oxidant sources. Therefore, taking into account the overall concentrations and compositions of antioxidants in lettuce, no single assay accurately reflects the mechanism of action of all antioxidants present in the plant (Prior *et al.* 2005). Furthermore, none of the works previously mentioned has studied the distribution of phytochemicals in butterhead lettuce type and growing evidence suggests that cultivar may alter the phytochemical content of lettuce (Ozgen and Sekerci 2011).

The main objectives of this work were to characterize the phytochemical content of butterhead lettuce at harvest and to evaluate how they are distributed in the whole plant. To achieve this goal, reduced ascorbic acid (AA), total chlorophyll (TC), total carotenes (TCar) and total phenolics (TP) contents were assessed according to a ring disposition. Also the browning potential was measured, in order to determine how the phytochemical content affects the enzymatic browning of lettuce. The antioxidant capacity of lettuce extracts was determined by plant zones using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity assay, the trolox-equivalent antioxidant capacity (TEAC) assay and the oxygen radical absorbance capacity (ORAC) assay. Additionally, the difference in individual phenolic compounds between outer and inner lettuce zones was carried out using high-performance liquid chromatography (HPLC) coupled with diode array detector.

MATERIALS AND METHODS

Plant Material and Sample Preparation

Heads of butterhead lettuce (*Lactuca sativa* var. Lores) were grown and harvested in Sierra de los Padres, Mar del Plata, Argentina during early spring of 2011 with an average temperature in the area of 11.9C (Servicio Meteorológico Nacional, 2013). Lettuce was cultivated under plastic covering house with standard conventional practices. Lettuce heads were hand-harvested after reaching a marketable size (approximately 24–30 leaves/head) and transported within 60 min to the laboratory. At each of the three experimental runs and for each index assayed, three whole plants were processed within 1–2 h after harvest.

In each plant, reduced AA, TC, TCar, TP and browning potential were measured in all the rings from the external ratio toward the internal one. A ring was constituted by leaves localized at the same growing point of the plant. Each lettuce ring had between two and four leaves, and the number of total rings per lettuce plant was about six. Numbers were used to identify each ring, starting with number 1, which corresponds to the first outer leaves (older leaves), and finishing with number 6, which corresponds to the inner leaves (younger leaves).

Antioxidant activity was assessed using three different methodologies: DPPH, TEAC and ORAC. These assays were determined by zones. In this sense, three different zones of the complete lettuce head, called external (outer leaves), middle (mid leaves) and internal zones (inner leaves), were analyzed. For each lettuce plant, zones were delimited visually, applying an organoleptic criterion, according to which the internal zone was compact with yellow leaves and the middle and external zones corresponded to noncompact leaves of green and dark green color, respectively. Each zone had a mean of approximately four to eight leaves.

For TC, total carotene (TCar) and TP contents, each ring was frozen in liquid N_2 and stored at −20C until analysis. AA content and browning potential were analyzed within 1–2 h after harvest using fresh material. To evaluate antioxidant activity indices and HPLC phenolics profile, each zone of the lettuce head was freeze-dried using a freeze dryer (Karaltay Scientific Instruments Co., Ltd., Beijing, China). Lyophilizes were stored at −20C until analyzed.

Reduced AA Content

Reduced AA content was determined following the titrimetric assay described by Roura *et al.* (2001). Samples (20 g) from each lettuce ring were extracted with 100 mL of metaphosphoric acid solution (60 g/kg) for 3 min using a commercial blender (Multiquick, MR 5550 CA Braun, Espanola S.A., Barcelona, Spain) with a homogenizer speed of 3500–7000 rpm. The homogenate was made up to 250 mL with 30 g/kg metaphosphoric acid and filtered through Whatman No. 42 filter paper (GE Healthcare Life Sciences, Piscataway, NJ). Temperature during AA extraction was maintained at 0C. Two or three aliquots (10 mL each) of the filtrate were titrated independently with 2,6-dichloroindophenol. The AA of each lettuce ring was reported on a dry basis: mg AA/g dry weight (DW).

TC and Carotenoids Content

Chlorophyll and carotenoids contents were determined according to Roura *et al.* (2001). Frozen samples from each lettuce ring were processed individually in a small cryogenic mill (SPEX SamplePrep 6770 Freezer/Mill, Metuchen, NJ). One gram of powdered sample was mixed with 20 mL of cold acetone and stirred during 3 h at 0C in an orbital shaker at 100 rpm. The acetone extract was added to 20 mL of freshly distilled diethyl ether and washed three times with a 10% sodium chloride solution. This homogenate was filtered through sintered glass and water was removed from the filtrate with anhydrous sodium sulfate. Absorbance of the filtrate at 660.0 and 642.5 nm was measured for chlorophyll determination and at 450 nm for carotene analysis with a ultraviolet (UV) 1601 PC UV-visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). TC content was reported as mg TC/g DW and TCar content was expressed as mg TCar/g DW.

TP Content

TP content was determined spectrophotometrically using the Folin–Ciocalteu reagent (FCR) according to the methodology proposed by Singleton *et al.* (1999) with modifications. Frozen samples from each lettuce ring were processed individually in a small cryogenic mill (SPEX SamplePrep 6770 Freezer/Mill) and 2 g of the resulting powder were mixed with 6 mL of ethanol and stirred during 3 h at 0C in an orbital shaker at 100 rpm. The mixture was centrifuged at 10,000 rpm for 15 min. A sample of the extract (200 μL) was added to 1,000 μL of FCR (diluted 1/10). After 3 min of incubation at ambient temperature, 800 μL of 7.5% Na_2CO_3 solution was added and the reaction mixture was incubated for 2 h at the same temperature. The absorbance was measured at 765 nm and TP content was calculated using gallic acid (GA) as standard. Results were expressed as mg GA/g DW.

Phenolics Extraction for HPLC Analysis and Antioxidant Activity Assays

Freeze-dried samples from each lettuce zone (0.2 g) were homogenized with 10 mL solution of methanol (80%). The homogenate was sonicated for 30 min in a Bransonic 2210 sonicator (Bransonic Ultrasonic Co., Danbury, CT) and then centrifuged at 14,000 rpm for 15 min at 4C (Beckman Coulter Centrifuge, Allegra 64R, Beckman Coulter, Palo Alto, CA). The supernatant was collected and the precipitate was extracted again with 10 mL of 80% methanol, under the conditions previously described. The two supernatants were mixed and filtered using Whatman filter paper no. 1. The volume of pooled supernatant was brought up to 12.5 mL. The final methanolic extract was stored at −25C to be used in the determination of DPPH, TEAC and ORAC assays and for the determination of the HPLC phenolics profile of lettuce.

Antioxidant Activity Assays

DPPH Scavenging Assay. The DPPH assay was conducted according to the method reported by Brand-Williams *et al.* (1995) with some modifications. DPPH solution was prepared by mixing 2.5 mg of DPPH radical with 100 mL of pure methanol. The solution was adjusted at an absorbance of 1.0 ± 0.02 at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic) was used as a standard and 80% methanol was used as a blank. Samples of 20 μL of the lettuce extract were placed in a microplate and 280 μL of DPPH radical were added. The mixture was kept in the dark for 30 min. The absorbance was read using an Omega spectrophotometer (BMG Labtech Inc., Deckenpfronn, Germany) with a microplate reader device, at a wavelength of 515 nm. The percentage of inhibition was calculated for each sample, which indicates the capacity of the antioxidants to reduce the absorbance of the radical after incubation time. A calibration curve was prepared using an aqueous solution of trolox and results were expressed as μmol of trolox equivalents (TE)/g DW.

TEAC Assay. This assay is based on the ability of the antioxidants to scavenge the blue–green $ABTS^{\bullet+}$ radical cation relative to the $ABTS^{\bullet+}$ scavenging ability of the water-soluble vitamin E analog Trolox. TEAC value was determined according to Re *et al.* (1999). $ABTS^{\bullet+}$ cation was generated through the interaction of 19.2 mg of ABTS (2′2-azinobis(3-ethylbenzotriazoline-6-sulfonic acid)), dissolved in 5 mL of HPLC-grade water and 88 µL of potassium persulfate ($K_2S_2O_8$; 0.0378 g/mL). It was incubated in the dark at room temperature for 16 h; then 1 mL of ABTS-activated radical was taken and 88 mL of ethanol was added. The radical was adjusted at an absorbance of 0.7 ± 0.02 at 734 nm. The reaction was initiated adding 245 µL of $ABTS^{\bullet+}$ and 5 µL of the extract or trolox standard solution in methanol and absorbance was monitored at 734 nm at 1 and 6 min in an Omega spectrophotometer (BMG Labtech Inc.) with a microplate reader device. The percentage of inhibition was calculated and the results were expressed as µmol TE/g DW.

ORAC Assay. This assay measures the effect of antioxidant components in fruits and other foods on the decline in fluorescence of fluorescein induced by a peroxyl radical generator. The lettuce extracts were subjected to the ORAC assay as described by Alvarez-Parrilla *et al.* (2010) with minor modifications. Briefly, 20 µL of appropriately diluted sample or trolox standard was mixed with 120 µL fluoresceine (80 nM) and 60 µL AAPH (2,2′azobis (2-amidino-propane) dihydrochloride) (40 mM). Fluorescence was measured every 2 min for 60 min in a FLx800tbi plate reader (Bio-Tek Instruments, Winooski, VT), with excitation and emission filters of 485/20 and 528/25, respectively. Results were expressed as µmol TE/g DW.

HPLC Analyses for Phenolics

The lettuce extracts (20 µL) were analyzed using an HPLC system equipped with a pump and a SPD-M 20A photodiode array UV-VIS detector (Shimadzu Prominence). Separations were achieved on a Gemini C18 column (250 × 4.6 mm, 5 µm, Phenomenex, Torrance, CA). The mobile phases were phosphoric acid 0.1% (v/v; A) and acetonitrile:phosphoric acid (99.9:0.1 v/v; B) with a solvent flow rate of 1.2 mL/min. The gradient program started at 15% B in A, reaching 30% B at 12 min, and then 80% B at 20 min. After equilibration for 1 min at 80% B, the composition of solution returned to the initial condition (15% B). Chromatograms were recorded at 280 and 325 nm. Spectra were recorded from 190 to 380 nm. The main phenolic compounds of inner and outer lettuce leaves were identified and quantified by means of their UV spectrometric data and retention times and by comparison with standard compounds (0.4 mg/100 mL) such as chlorogenic acid, caffeic acid, chicoric acid, quercetin and kaempferol (all from Sigma–Aldrich, St. Louis, MO) and with previously published chromatograms of lettuce extracts (Hohl *et al.* 2001; Romani *et al.* 2002; Llorach *et al.* 2008; Li *et al.* 2010; Ribas-Agustí *et al.* 2011; Mai and Glomb 2013).

Browning Potential

Overall browning potential was measured as the absorbance of an aqueous extract of lettuce leaves at 320 nm (Pereyra *et al.* 2005). Briefly, leaf tissue from each lettuce ring was homogenized with distilled water (1:20) using a tissue homogenizer (Braun, Kronberg, Germany) with a speed of 3,500–7,000 rpm. The homogenate was filtered through Whatman N°42 filter paper. The cloudy supernatant was centrifuged at 10,000 rpm for 15 min. The absorbance of a supernatant aliquot was measured with a spectrophotometer at 320 nm.

Statistical Analysis

Results reported in this paper are LSMEAN values (least square mean, estimators of means by the method of least squares) together with their standard deviations. Data were analyzed using R, software version 2.12 (R Development Core Team 2011). Probability level was fixed to $P < 0.05$.

The factors employed as sources of variation were RING (one to six) for reduced AA, chlorophyll content, total carotenoids content, TP content and browning potential assays and ZONE (external, middle and internal) for antioxidant activity assays.

Differences among results obtained for different factors levels were evaluated with the multiple comparisons Tukey–Kramer test ($P < 0.05$; Kuehl 2001). Correlation analyses between AA content, TC content, total carotenoids content, TP content and antioxidant activity assays (DPPH, TEAC and ORAC) were carried out through Pearson's coefficients evaluation. To correlate data between indexes evaluated by zones and by rings, those indexes evaluated by rings were recalculated according to a zone distribution, where rings one and two corresponded to the external zone, rings three and four to the middle zone, and rings five and six to the internal zone. Correlations of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Reduced AA Content

Figure 1A shows the profiles obtained for AA content in lettuce heads as a function of leaf ring number. Significant differences ($P < 0.01$) in AA were found between developed

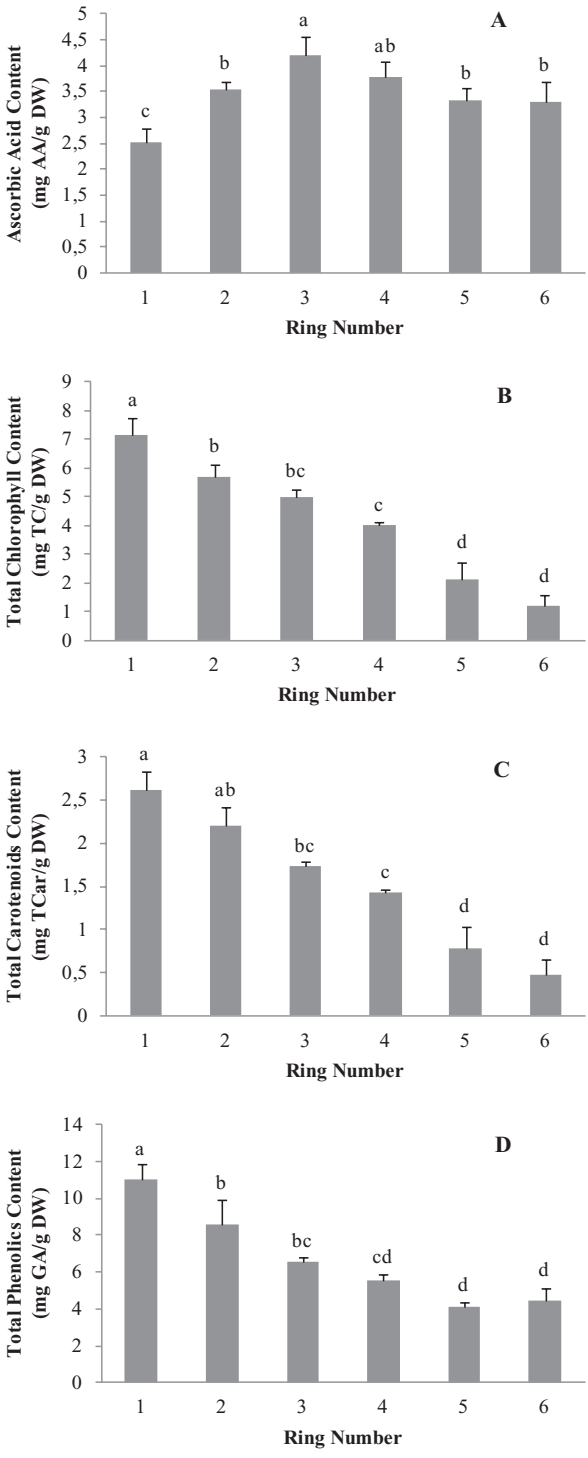

FIG. 1. ASCORBIC ACID CONTENT (A), TOTAL CHLOROPHYLL CONTENT (B), TOTAL CAROTENOIDS CONTENT (C) AND TOTAL PHENOLICS CONTENT (D) PROFILE IN BUTTERHEAD LETTUCE AS A FUNCTION OF LEAF RING NUMBER (FROM RING NUMBER ONE: OUTER LEAVES, TO RING NUMBER SIX: INNER LEAVES). DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES BETWEEN THE VALUES ($P < 0.05$)

and undeveloped leaves. AA distribution showed an increase in AA of 66% from rings one to three (2.54 ± 0.27, 3.56 ± 0.15, 4.22 ± 0.34 mg AA/g DW for rings one, two and three, respectively), a constant content between rings three and four (3.79 ± 0.30 mg AA/g DW) and then a gradual decrease for the other rings, reaching rings five and six similar values to that of ring two. Goñi *et al.* (2010) working with greenhouse butterhead lettuce reported a similar profile for AA content. Siomos *et al.* (2002) also reported that middle leaves of romaine and crisphead lettuce types had the highest AA, but for lettuce of the leaf type no significant difference in AA relative to leaf position was observed.

The fact that the external ring had a lower AA content than the others could be due to a higher exposition of these outer leaves to environmental conditions (Goñi *et al.* 2010). Moreover, these leaves were laid down on the soil so they were more exposed to soil microorganisms' action, irrigation water and residual metabolites of agrochemicals. These factors acting alone or in combination could accelerate the AA degradation of this more exposed leaf ring (Hancock and Viola 2005). Also the oxidized form of vitamin C, dehydroascorbic acid, generally constitutes a larger proportion of the total vitamin C in the older leaves. This may reflect a lower capacity of older plants to reduce dehydroascorbic acid to AA (Hodges and Forney 2003; Toledo *et al.* 2003). AA has a higher reducing capacity and antioxidative potential than dehydroascorbic acid. In this sense, the antioxidative capacity supplied by vitamin C decreased from rings three to one (Fig. 1A). This may have physiological implications, both for the plant and its defense against oxidative stress, and for human health when lettuce is ingested in the diet (Hancock and Viola 2005).

In inner undeveloped leaves (rings five and six), the lower AA could be attributed to differences in the maturity state of the leaves. Additionally, although light is not essential for the synthesis of AA in plants, the amount and intensity of light have a definite influence on the amount of AA formed. AA is synthesized from sugars supplied through photosynthesis in plants. Fruits and vegetables exposed to maximum sunlight contain higher amounts of vitamin C than fruits and vegetables on the same plant that are indoors and shaded. It is also known that those tissues more exposed to environmental factors such as light have more AA content to protect the vegetable from outside stress (Lee and Kader 2000). Goñi *et al.* (2010) and Agüero *et al.* (2011) also found lower values of AA in inner leaves of butterhead lettuce. However, Guerra *et al.* (2010), working with celery, reported that undeveloped inner leaves presented the highest AA content. This discrepancy may be due to the different vegetable crop used in the studies as well as differences in growth habit and growing conditions.

TC and Carotenoids Content

Analysis of variance (ANOVA) applied on TC and carotenoids content data showed great differences ($P < 0.001$) in both pigment concentrations among lettuce rings. As expected, a gradual decrease of chlorophyll and carotenes contents was observed from external to internal rings (Fig. 1B,C), with extreme values of 7.13 ± 0.63 and 1.18 ± 0.38 mg TC/g DW for rings one and six, respectively for chlorophyll content, and 2.61 ± 0.21 and 0.47 ± 0.18 mg TCar/g DW for rings one and six, respectively for carotenes contents. This behavior is associated to differences in maturity and sun exposure among the leaves of the plant. External rings of lettuce heads are constituted by mature and developed leaves, characterized by a dark green color because of its high chlorophyll content. In contrast, inner rings have young and undeveloped leaves that exhibit a yellow color because of its lower content of chlorophyll (Agüero *et al.* 2008). Additionally, the higher exposure of outer leaves to sunlight induces the synthesis of chlorophyll and also of carotenoids because carotenoids are closely attached to the chlorophyll molecules to remove excess energy from the photosynthetic system to prevent damage (Marschner and Rimmington 1996; Taiz and Zeiger 1998; Tracewell *et al.* 2001). Several authors have also studied the impact of the tissue age of different vegetables on the content of these pigments, finding similar results than those reported here (Yoo *et al.* 2003; de Azevedo and Rodriguez-Amaya 2005; Ozgen and Sekerci 2011). Agüero *et al.* (2008), working with three different zones of the complete lettuce head (external, middle and internal zones), reported comparable relations between TC and leaf development, being values in external leaves higher than in internal ones.

TP Content

Concentration of phenolic compounds was significantly influenced by leaf age and position (Fig. 1D). The highest TP content corresponded to the most photosynthetic lettuce leaves, the outermost ones (11.00 ± 0.86 mg GA/g DW), while the lowest was for rings five and six (4.09 ± 0.26 and 4.45 ± 0.67 mg GA/g DW, respectively). Middle leaves of the lettuce head presented intermediate values with a TP content for rings two, three and four of 8.59 ± 1.34, 6.58 ± 0.19 and 5.51 ± 0.39 mg GA/g DW, respectively.

It has been demonstrated that antioxidants in plants are part of a complex defense mechanism against a wide range of stresses and thus, accumulate in response to these stresses (Dixon and Paiva 1995; Blokhina *et al.* 2003). As phenolics are one of the major groups that contribute to the antioxidant activity of lettuce, it is consistent that outer leaves, which had been more exposed to environmental and stressful conditions than the other leaves of the plant presented higher TP content. These results are also in agreement with those reported by Ozgen and Sekerci (2011) who informed significantly higher TP content in lettuce outer leaves with respect to middle and inner leaves in both red and green color lettuces. These authors reported that the magnitude of differences between the leaves positions was fourfold in red lettuce while outer leaves of green cultivars contained only 70% higher TP than inner leaves. In the present work, the TP of outer leaves, constituted by rings one and two was 38% and 56% higher with respect to middle (rings three and four) and inner leaves (rings five and six), respectively. Cano and Arnao (2005) also reported that phenolics content of romaine lettuce increased from stem to outermost leaves, passing through inner and medium leaves, although no significant changes were observed in iceberg or baby head lettuces in this respect. However, Pandjaitan *et al.* (2005) reported for mid-maturity spinach leaves higher levels of TP, total flavonoids and antioxidant capacity than immature and mature leaves. These differences may be due to various factors such as type of vegetable, and pre- and postharvest conditions.

Antioxidant Activity

While for DPPH and TEAC assays significant differences ($P < 0.05$) in the antioxidant activity among the three lettuce zones were observed, ANOVA applied on ORAC data showed no significant differences with respect to ZONE factor (Table 1). Additionally, values obtained with ORAC technique were 4 and 14 times higher than with TEAC and DPPH assays, respectively. Tabart *et al.* (2009) reported that antioxidant activities of standard antioxidants measured by the ORAC assay were considerably higher than those of the TEAC and DPPH values, as it was found in this work. The most important naturally occurring plant substances showing antioxidant activity are carotenoids, flavonoids and other simple phenolic compounds, besides vitamins A, C and E (Cano and Arnao 2005). The ORAC assay measures

TABLE 1. ANTIOXIDANT ACTIVITY OF LETTUCE ZONES DETERMINED BY DPPH, TEAC AND ORAC METHODS

Zone	DPPH*	TEAC*	ORAC*
External	11.82 ± 1.08^{a}	36.03 ± 5.36^{a}	113.16 ± 3.05^{a}
Middle	7.84 ± 0.63^{b}	29.93 ± 4.55^{ab}	115.81 ± 1.65^{a}
Internal	5.68 ± 1.20^{c}	29.32 ± 3.47^{b}	115.43 ± 0.45^{a}

* Values are μmol TE/g dry weight.

[abc] Mean values with different letter within the same column are significant different ($P < 0.05$).

DPPH, 2,2-diphenyl-1-picrylhydrazyl; TE, trolox equivalents; TEAC, trolox-equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity.

all traditional antioxidants including hydrophilic and lipophilic antioxidants. Additionally, ORAC results reflect more than just radical scavenging because it is the only method combining both an inhibition time and a degree of inhibition (Cao *et al.* 1997). These characteristics of the ORAC assay may explain the higher values obtained with this method with respect to DPPH and TEAC methodologies.

The results obtained with DPPH technique clearly indicate the potential of the outer leaves extracts to scavenge free radicals. For this index, outer leaves had twice the antioxidant activity than internal ones, while middle leaves had intermediate values. TEAC results also expressed significant higher antioxidant activity in the external and middle zones of lettuce, with the lower capacity corresponding to inner zone. These results are in agreement with those mentioned earlier that outer leaves accumulate more antioxidant compounds as a result of their higher exposure to environmental and stressful conditions. Additionally, our findings are consistent with previous reports on antioxidant activity of other varieties of green and red lettuces (Cano and Arnao 2005; Ozgen and Sekerci 2011).

Correlation Analysis

Table 2 lists Pearson's coefficients for all cross correlations among parameters analyzed: AA content, TC content, total carotenoids content (TCar), TP content and antioxidant activity assays (DPPH, TEAC and ORAC).

Phenolics content was strongly correlated with TP ($r = 0.801$, $P < 0.01$) and total carotenoids ($r = 0.801$, $P < 0.01$). This is in agreement with the fact that the synthesis of phenolics are directly related with the photosynthetic activity of tissues (Furuya 1993; Short and Briggs 1994).

TABLE 2. PEARSON CORRELATION COEFFICIENTS (r) BETWEEN INDEXES OF PHYTOCHEMICAL CONTENT: ASCORBIC ACID CONTENT (AA), TOTAL CHLOROPHYLL CONTENT (TC), TOTAL CAROTENOIDS CONTENT (TCar), TOTAL PHENOLICS CONTENT (TP) AND ANTIOXIDANT CAPACITY (DPPH, TEAC AND ORAC)

	AA	TC	TCar	TP	DPPH	TEAC	ORAC
AA	1.000						
TC	–0.206	1.000					
TCar	–0.245	0.998[c]	1.000				
TP	–0.233	0.801[b]	0.801[b]	1.000			
DPPH	–0.424	0.835[c]	0.857[c]	0.755[b]	1.000		
TEAC	–0.476	0.081	0.099	0.193	0.132	1.000	
ORAC	–0.010	0.473	0.481	0.301	0.541[a]	–0.107	1.000

[a] $P < 0.1$ (correlated).

[b] $P < 0.01$ (strongly correlated).

[c] $P < 0.001$ (very strongly correlated).

DPPH, 2,2-diphenyl-1-picrylhydrazyl; TE, trolox equivalents; TEAC, trolox-equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity.

Moreover, TC was very strongly correlated with total carotenoids ($r = 0.998$, $P < 0.001$) evidencing that carotenoids are closely attached to the chlorophyll molecules to remove excess energy from the photosynthetic system and prevent tissue damage.

Antioxidant activity by DPPH method was very strongly correlated with TC ($r = 0.835$, $P < 0.001$) and carotenoids contents ($r = 0.857$, $P < 0.001$), which means that these compounds contribute generously with the radical-scavenging capacity of the lettuce head.

TEAC and DPPH methods assess antioxidant activity through reduction of the radicals by a hydrogen-donating antioxidant (Prior *et al.* 2005). Additionally, it is known that the Folin–Ciocalteu method, used to assay the TP content of the lettuce extracts, also measures the total reducing capacity of the sample. So, TP generally correlates with redox and antioxidant capacities measured by TEAC and DPPH methods (Tabart *et al.* 2007). In this work, phenolics were strongly correlated with DPPH method ($r = 0.755$, $P < 0.01$), but not with TEAC and ORAC assays. However, results of TEAC assay showed that outer lettuce leaves presented higher antioxidant activity, which is in consonance with the higher phenolics content found in these leaves.

Phenolic Compounds Identification and Quantification

The identification of phenolic compounds was carried out on the outer and inner leaves of butterhead lettuce, since middle leaves present intermediate phenolics content between older and younger leaves, as was previously demonstrated. The major phenolic compounds identified in the two lettuce zones analyzed were the phenolic acids chicoric (di-O-caffeoyltartaric acid), chlorogenic (5-O-caffeoylquinic acid) and isochlorogenic (3,5-di-O-caffeoylquinic acid) (Fig. 2A,B and Table 3). Two quercetin glycosides were also recognized, which were tentatively identified as quercetin-3-O-glucoside and quercetin-3-O-glucuronide by comparing data with previous reports found in lettuce (Hohl *et al.* 2001; Romani *et al.* 2002; Llorach *et al.* 2008; Ribas-Agustí *et al.* 2011; Mai and Glomb 2013). Minor amounts of caffeic acid and hydroxicinnamic acid derivate were also found. None of the lettuce zones have measurable quantities of the aglycone quercetin and kaempferol. According to several authors, phenolic acids are predominant compounds in green leaf lettuce, mainly di-O-caffeoyltartaric acid and 5-O-caffeoylquinic acid, with minor amounts of glycosilated flavonoids (Romani *et al.* 2002; Llorach *et al.* 2008; Oh *et al.* 2009; Li *et al.* 2010; Ribas-Agustí *et al.* 2011). Our findings are in concordance with those studies. Moreover, the identified phenolic compounds coincide with those reported by Romani *et al.* (2002) for the lettuce variety Audran.

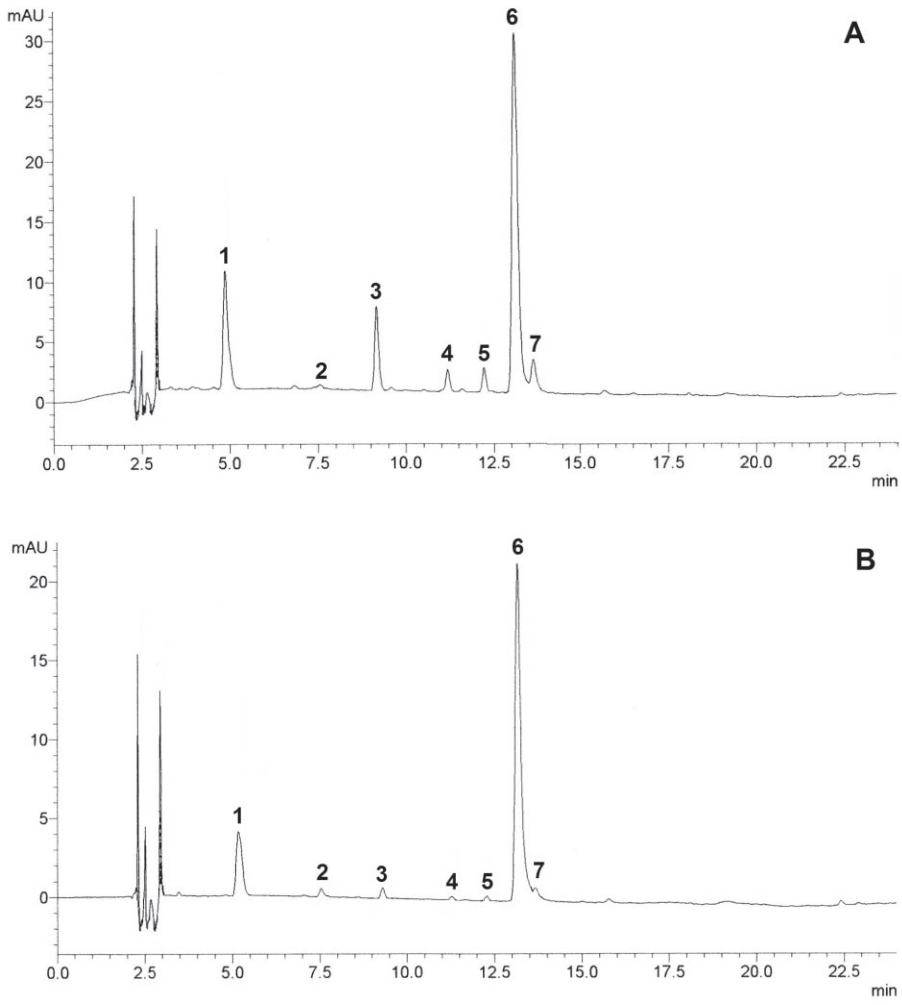

FIG. 2. CHROMATOGRAPHIC PROFILE ACQUIRED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–DIODE ARRAY DETECTOR (325 NM) OF OUTER (A) AND INNER (B) BUTTERHEAD LETTUCE LEAVES. PEAKS: 1, CHLOROGENIC ACID; 2, CAFFEIC ACID; 3, ISOCHLOROGENIC ACID; 4, QUERCETIN-3-O-GLUCOSIDE; 5, QUERCETIN-3-O-GLUCURONIDE; 6, CHICORIC ACID; 7, HYDROXICINNAMIC ACID DERIVATE

The two lettuce zones showed almost identical phenolic profile as can be observed in the HPLC chromatograms of Fig. 2A,B. However, significant differences were detected in the content of the major peaks. The three major phenolic acids (chicoric, chlorogenic and isochlorogenic) accounted for 91% and 97% of total phenols in external and internal zones, respectively. Minor peaks corresponding to caffeic acid, the two quercetin glycosides and the hidroxycinnamic acid derivate, represent the remaining 9% and 3%, for external and internal zone, respectively. The difference between both zones lies in the content of each phenolic compound. Thus, chicoric acid and chlorogenic acid content in the outer leaves was 0.5 and twofold greater relative to the inner leaves, respectively, while for isochlorogenic acid the difference was much higher (11-fold higher for outer leaves compared with inner). Also for the other minor hydroxicinnamic acid derivate, greater content was found in the external zone except for caffeic acid, in which inner

TABLE 3. MAIN PHENOLIC ACIDS IN THE OUTER AND INNER ZONES OF BUTTERHEAD LETTUCE

Zone	Chicoric acid*	Chlorogenic acid*	Isochlorogenic acid*	Caffeic acid*
External	1930.4	822.8	340.6	6.1
Internal	1280.8	398.4	30.6	10.4

* Values are μg/g dry weight.

leaves presented nearly twice the content of outer leaves. In the case of flavonoids, both quercetin glycosides were seven times higher in the outer zone than in the inner. These findings are in concordance with the fact that outer lettuce leaves presented higher TP content and also browning potential. Because caffeoyl derivatives are the most important substrates for enzymatic browning (Mai and Glomb 2013), it is consistent than the outer lettuce zone, which presented a higher content of these compounds also showed higher browning potential. Additionally, the biosynthesis of flavonoids depends on light, which explains the major accumulation of quercetin glycosides in the outer leaves of the plant (Hohl *et al.* 2001).

Browning Potential

One of the most common postharvest disorders of lettuce is the browning of the stem, which greatly reduces the visual quality of the plant. Additionally, the photosynthetic lettuce tissue also enables browning, although other natural pigments such as chlorophyll mask the discoloration. This phenomenon is caused by the action of the enzyme polyphenol oxidase (PPO) that catalyzes the oxidation of phenolic compounds to *o*-quinones. Then the *o*-quinones condense with other compounds and form brown polymers responsible of browning. It is known that a range of phenolics may be degraded by the PPO and several authors have studied the implication of this enzyme in lettuce (Tomás-Barberán *et al.* 1997).

Figure 3 depicts the evolution of browning potential index with respect to leaf age and position. Browning potential of rings one and two were almost twice with respect to ring six; this means that older and developed leaves showed a high tendency to suffer enzymatic browning reactions and thus a shorter potential shelf life. Additionally, it is consistent with the fact that accumulation of phenolic compounds induced tissue browning because outer leaves presented both higher phenolics content and browning potential.

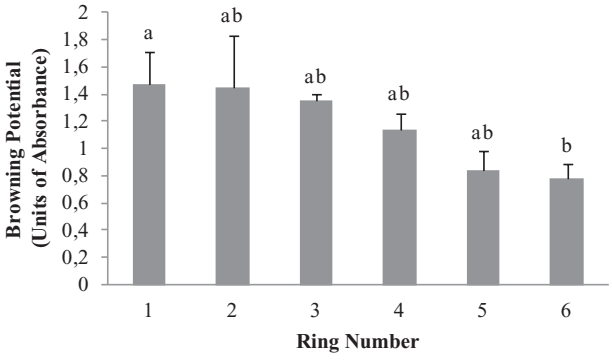

FIG. 3. BROWNING POTENTIAL PROFILE IN BUTTERHEAD LETTUCE AS A FUNCTION OF LEAF RING NUMBER. (FROM RING NUMBER ONE: OUTER LEAVES TO RING NUMBER SIX: INNER LEAVES). DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES BETWEEN THE VALUES ($P < 0.05$)

Lettuce is a usual raw material for minimal processing salads. Wounding during the preparation of fresh cut lettuce induces the synthesis of enzymes of phenylpropanoid metabolism and the synthesis and accumulation of phenolics with subsequent tissue browning (Tomás-Barberán *et al.* 1997). In this sense, there is a point of non-coincidence between the pursuit of a minimally processed product with a long shelf life and fresh appearance and the need to follow a diet rich in bioactive compounds. From a nutritional standpoint, the external zone of lettuce is rich in bioactive compounds (carotenes, chlorophylls and phenolics), which contribute with a high antioxidant capacity, but the presence of phenolics also contribute to the enzymatic browning of leaves decreasing its potential shelf life.

CONCLUSION

Leaf age and position had been proven to have an impact on the composition of phytochemicals of butterhead lettuce at the moment of harvest. However, not all phytochemicals tested in this work behaved similarly. The external zone of lettuce constituted by older lettuce leaves proved to be rich in some bioactive compounds (phenolics, chlorophyll and carotenoids), which contribute with a high antioxidant capacity as determined by DPPH and TEAC methods. However, the presence of phenolics in these leaves also contributes to the enzymatic browning. On the other hand, the outer oldest leaves also presented at harvest the lowest AA content.

This study presents for the first time the identification and quantification of polyphenols in butterhead lettuce variety Lores in relation to leaf age and position in conjunction with a thoroughly description of the distribution of other major phytonutrient compounds and antioxidant activity. Evidently the outer leaves much more exposed to environmental conditions, with higher chlorophyll content and a metabolism nearly senescence require higher content of phytochemicals to give resolution to different biological and physical stresses.

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