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Effect of leaf position on the distribution of phytochemicals and antioxidant capacity among green and red lettuce cultivars

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Abstract

In recent years, there has been an increasing interest in consumption of red fruits and vegetables due to its rich dietary sources of antioxidant, phenolics and anthocyanins. In this study, phytochemical and antioxidant properties of green and red lettuce cultivars were studied. Particularly, the effect of leaf position on these properties was compared. Eight conventionally grown cultivars of lettuce (*Lactuca sativa* L.), with four green (Fonseca, Freckles, Krizet and Filipus) and four red (Versai, Nation, Paradai and Cherokee) cultivars were studied. Their leaves were divided into three groups; outer, middle and inner part. Total antioxidant capacity (TAC) of each group of leaves was assessed by both trolox-equivalent antioxidant capacity (TEAC), and the ferric reducing antioxidant power (FRAP) assay. Leaf color, total soluble solids, total phenolics (TP), total anthocyanin (TACY), chlorophyll a and b were determined. The results demonstrated that outer leaves have the highest phytonutrient content and antioxidant properties. Outer leaves exhibited significantly higher TP and TAC than middle and inner leaves in both red and green color lettuce. The average TP content of red lettuce were 845, 297 and 195 µg gallic acid equivalent per gram fresh weight in the outer, middle and inner leaves, respectively. Also TAC of outer leaves was significantly higher than middle and inner leaves in both red and green color lettuce. The magnitude of difference between red and green cultivars was significant as determined by both TEAC and FRAP methods.

Additional key words: anthocyanin; chlorophyll; FRAP, *Lactuca sativa*; phenolic; TEAC; vegetable.

Resumen

Efecto de la posición de la hoja en la distribución de los productos fitoquímicos y la capacidad antioxidante entre cultivares verdes y rojos de lechuga

En los últimos años ha habido un interés creciente por consumir frutas y verduras de color rojo, por ser una fuente rica de antioxidantes, polifenoles y antocianinas. En este trabajo se estudiaron las propiedades fitoquímicas y antioxidantes de cultivares de lechuga verdes y rojos, comparando, en particular, el efecto de la posición de la hoja sobre estas propiedades. Se estudiaron ocho cultivares convencionales de lechuga (*Lactuca sativa* L.), cuatro verdes (Fonseca, Freckles, Krizet y Filipus) y cuatro rojos (Versai, Nation, Paradai y Cherokee), dividiendo sus hojas en tres grupos: externas, medias e internas. Se evaluó la capacidad antioxidante total (TAC) de cada grupo de hojas, tanto la capacidad antioxidante equivalente de Trolox (TEAC), como el ensayo de potencia antioxidante reductora férrica (FRAP). También se determinó el color, los sólidos solubles totales, fenoles totales (TP), el total de antocianinas (TACY), y las clorofilas a y b de las hojas. Los resultados mostraron que las hojas externas tenían un mayor contenido de fitonutrientes y antioxidantes, así como TP y TAC significativamente mayores que las hojas medias e internas, tanto en lechugas rojas como verdes. El contenido promedio de TP en las lechugas rojas fue 845, 297 y 195 195 µg de equivalentes de ácido gálico por gramo de peso fresco en las hojas exteriores, medias e internas, respectivamente. También el TAC de las hojas externas fue, en ambos tipos de lechuga, significativamente mayor que el de las hojas medias e internas. La diferencia entre los cultivares rojos y verdes fue significativa tanto con los métodos TEAC como FRAP.

Palabras clave adicionales: antocianinas; clorofila; fenólicos; FRAP; hortícolas; *Lactuca sativa*; TEAC.

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Abbreviations used: FRAP (ferric reducing antioxidant power); fw (fresh weight); GAE (gallic acid equivalent); TAC (total antioxidant capacity); TACY (total anthocyanin); TE (trolox equivalent); TEAC (trolox equivalent antioxidant capacity); TSS (total soluble solid); TP (total phenolic).

Introduction

Researchers have long studied plant pigments in fruits and vegetables due to their vital role in visual appeal. More recently, efforts have included nutritional evaluation and improvement of crops as a source of phytonutrients (Özgen *et al.,* 2009). Evidence that anthocyanins have beneficial effects for human health are increasingly being reported in the scientific literature and these compounds are now widely recognized as potential remedial compounds (Hou, 2003; Lila, 2004; Ghosh, 2005). Anthocyanins, which act as powerful antioxidants, are increasingly being shown helping to optimize human health by neutralizing harmful free radicals in the body. These antioxidants reduce oxidative damage by cells that can lead to cancer, heart disease, and other degenerative diseases (Hou, 2003; Prior, 2003). Recent trends show that people are selecting and consuming more anthocyanin rich red fruits and vegetables. A good example of this is the increased consumption and high value of red lettuce compare to green lettuce. Lettuce is an important dietary leafy vegetable that is mostly consumed fresh, especially in salads. The health benefits of lettuce have been attributed to the presence of phenolic compounds, fiber and vitamin C content. Nicolle *et al.* (2004), found that a regular intake of antioxidant compounds from lettuce is useful to improve the lipid status and to prevent lipid peroxidation in tissues.

There are many different lettuce cultivars that range in color from green and yellow to deep red as a result of different concentrations of chlorophyll and anthocyanin in the leaves. Identification and quantification of anthocyanins, phenolics and antioxidant properties of green and red lettuce have been previously studied and researchers found that red lettuce has higher total phenolic (TP) and antioxidant capacity than green lettuce (Ferreres *et al.,* 1997; Zhao *et al.,* 2007; Llorach *et al.,* 2008). The primary difference between red and green lettuce is anthocyanin content. Mulabagal *et al.* (2010) compared *in vitro* biological activity of green and red lettuce and showed that the water extract of the red lettuce displayed higher biological activity and contained more anthocyanins in comparison to green cultivars. The HPLC profile of the red lettuce extract showed only one major anthocyanin and it was characterized as cyanidin-3-O-6-malonyl-β-glucopyranoside. There are also some studies that show the distribution of phytochemicals in head lettuce, some of which either account for the antioxidant potential of lettuce or closely related substances (Drews *et al.,* 1995; Hohl *et al.,* 2001). Most of these studies are focused on only green leaf lettuce (Drews *et al.,* 1995; Hohl *et al.,* 2001; Cano and Arnao, 2005). However, there is little information on the distribution of phytochemicals by leaf positions within these groups of green and red lettuce cultivars grown under the same environment. Growing evidence suggests that cultivar, planting date and growing conditions may alter the phenolic content and antioxidant capacity of lettuce (Liu *et al.,* 2007; Tsormpatsidis *et al.,* 2008). Diversity in tissue physiology, pigment forms and distribution leave open the possibility that phytochemicals and antioxidant capacity in lettuce may differ from outer to inner leaves. Furthermore, consumers tend to eat from middle part of lettuce head towards to inner since these parts look fresher, crispier and tender without knowing the phytonutrient contents of these parts. Therefore, we conducted this study to compare and contrast distribution of phytochemicals and antioxidant capacity affected by leaf position in both red and green lettuce cultivars that are grown under the same environmental conditions.

Material and methods

Plant material

Eight varieties of lettuce (*Lactuca sativa* L.) representing a range of pigmentation from dark red to green, four green (Fonseca, Freckles, Krizet and Filipus) and four red cultivars (Versai, Nation, Paradai and Cherokee) were studied. The characteristics of these cultivars are presented in Table 1. These cultivars were planted in early summer of 2008 at the Agriculture Research Station of Gaziosmanpasa University in Tokat (Turkey). Seedlings were grown 6 weeks in a climate-controlled greenhouse, and hardened off before planting in the field. At target amount of N (200 kg ha^{-1}) was applied from ammonium sulfate before transplanting the seedlings. The transplants were grown in the field with standard conventional practices. Cultivars were hand harvested and transported within 30 min to laboratory where the samples were processed.

Sample preparation

The whole lettuce plant was divided into three parts for each cultivar; outer one third of lettuce leaves

Table 1. Characteristics of lettuce cultivars used in the study

Cultivar	Leaf color	Type	Days to harvest
Versai	Red	Oak leaf	57
Nation	Red	Leaf lettuce	59
Paradai	Red	Oak leaf	59
Cherokee	Red	Batavia	64
Fonseca	Green	Batavia	57
Freckles	Green	Speckled romaine	56
Krizet	Green	Oak leaf	56
Filipus	Green	Romaine	57

representing "outer", inner one third of lettuce representing "inner" and the leaves in between representing the "middle part". Three replicates were used for each cultivar and each group of leaves. Three randomly chosen lettuces were used for each replication. Each group of leaves was chopped separately and composite samples were taken for each cultivar and replication. Whole-leaf samples were placed on a white background and single readings were taken on the upper surface of each leaf around similar area with hanheld unit. The color was measured using a Minolta portable chromameter (Minolta, Model CR-300 Ramsey, NJ, USA) which provided CIE L*, a* and b* values. A chromameter describes color in three coordinates: L*, lightness, from 0 (black) to 100 (white); a*, from –60 (green) to +60 (red); and b*, from –60 (blue) to +60 (yellow). Total soluble solid (TSS) concentration of fresh- chopped lettuce tissue was determined with a digital hand refractometer (Atago, Model ATC-1E Kyoto, Japan). The rest of the samples were frozen immediately and stored in about 100 g batches at –40°C until analyzed. For each lettuce sample, three replicates were thawed at room temperature and homogenized in a standard food blender with water (1:1 w/v). Slurries were assayed for total phenolic, antioxidant and anthocyanin using following methodologies. All the data are expressed on a fresh weight (fw) basis. The tissue percent moisture values were similar among the cultivars, with average values of 93-95%.

Analytical procedures

Total phenolic (TP) content was measured according to Singleton and Rossi (1965) procedure. Briefly, 1 mL slurries were extracted with buffer containing acetone, water, and acetic acid (70:29.5:0.5 v/v). Samples were incubated for 24 h in the dark at 4°C with occasional agitation. Samples were replicated three times. Aliquots of acetone buffer extract were diluted with distilled water. After addition of Folin Ciocalteu reagent, the mixture was incubated for 8 min at room temperature. Then, 7% sodium carbonate was added. After 2 h, the absorbance was measured by an automated UV-vis spectrophotometer (Model T60U, PG Instruments) at 750 nm. Gallic acid was used as a standard. The results were expressed as µg gallic acid equivalent (GAE) g^{-1} fresh weight (fw) basis.

Chlorophyll a, b and anthocyanin content were measured according to Kleinhenz *et al.* (2003). Briefly, 3 g slurries were mixed with 15 mL acidified methanol (HCl/methanol; 1:99 v/v) solution. Samples were incubated 24 h in the dark at 4°C with occasional agitation. After incubation, 1 mL of liquid fraction transferred to a clean cuvette, brought to a volume of 3 mL in acidified methanol solution. Readings of absorbance at 420 nm chlorophyll a (Chla), 515 nm anthocyanin (TACY), and 650 nm chlorophyll b (Chlb) were taken using a UV-vis spectrophotometer (Model T60U, PG Instruments). Standart curves were developed using solutions containing laboratory grade Chla and Chlb (Sigma-Aldrich, St. Louis, MO, USA) and cyanidin 3-glucoside chloride. The results were expressed as µg anthocyanin (cyanidin based) or chlorophyll g^{-1} fresh weight (fw) basis.

Total antioxidant activity (TAC) was estimated by using two standard procedures (FRAP and TEAC assays) as suggested by Özgen *et al.* (2006). The same methanolic extraction was used for chlorophyll and anthocyanin to determine TAC of lettuce samples.

The ferric reducing ability of plasma (FRAP) was determined according to the method of Benzie and Strain (1996). Assay was conducted using three aqueous stock solutions containing 0.1 mol L^{-1} acetate buffer (pH 3.6), 10 mmol L^{-1} TPTZ [2,4,6-tris(2-pyridyl)-1,3,5-triazine] acidified with concentrated hydrochloric acid, and 20 mmol L^{-1} ferric chloride. These solutions were prepared and stored in the dark under refrigeration. Stock solutions were combined (10:1:1 v/v/v) to form the FRAP reagent just prior to analysis. A 200 μL of green lettuce slurry or 50 μL of red lettuce slurry were mixed with 2.95 mL of FRAP reagent. After 30 min, the absorbance of the reaction mixture was determined at 593 nm on a spectrophotometer. The results were expressed as μmol trolox equivalent (TE) g^{-1} fw.

For the standard TEAC assay, ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) was dissolved in acetate buffer and prepared with potassium persulfate as described in Özgen *et al.* (2006). The mixture was diluted in acidic medium of 20 mM sodium acetate buffer (pH 4.5) to an absorbance of 0.700 ± 0.01 at 734 nm for longer stability (Özgen *et al.,* 2006). For the spectrophotometric assay, a 200 μL of green lettuce slurry or 50 μL of red lettuce slurry were mixed with 2.95 mL of ABTS solution. After 10 min of incubation, the absorbance was determined at 734 nm on a spectrophotometer. The results were expressed as μmol TE g^{-1} fw.

Statistical analysis

Data were analyzed using SAS software and procedures (SAS, 2006). Means were calculated using PROC TABULATE while ANOVA tables were constructed using PROC GLM separately ANOVAs were calculated for leaf position, leaf color and cultivar, the variable tested,. All cultivars were subjected to ANOVA jointly without their color groups. Mean were separated by least significant difference (LSD) method at 5% significant level for ANOVAs with significant factors.

Results and discussion

Effect of leaf position in TP and TAC content

Total phenolic content (TP) and antioxidant capacity (TEAC and FRAP) of green and red lettuce cultivars were significantly affected by the leaf position (Table 2). Outer leaves exhibited significantly higher TP and TAC than middle and inner leaves in both red and green color lettuce where the differences were more distinct particularly in red color cultivars.

These results showed that TP and TAC distribution was more profound in the outer leaves than in inner leaves. More than four fold differences were observed in red lettuce while outer leaves of green cultivars contained only 70% higher TP than inner leaves. The average TP content of red lettuce were 845, 297 and 195 μg GAE g^{-1} fw in the outer, middle and inner leaves, respectively. The average TP content of whole red and green lettuce was 445.6 and 273.3 μg GAE g^{-1} fw respectively. Filipus (green) and Cherokee (red) displayed the highest TP content among the cultivars used in this study (Table 2). These results are consistent with previous reports on TP and TAC content of green and red lettuce (Liu *et al.,* 2007; Bunning *et al.,* 2010). Cano and Arnao (2005) also found the same distribution pattern for TP and TAC in green lettuce type. They emphasized both hydrophilic and lipophilic portion of TAC.

Similarly with the TP results, TAC of outer leaves was also significantly higher than middle and inner leaves of both red and green color lettuce (Table 2). The magnitude of differences between the leaf positions were greater in red cultivars as determined by both TEAC and FRAP methods. When each leaf position or overall TAC was measured, red cultivars always displayed higher TAC compared to green cultivars. As some of the previous studies (Celik *et al.,* 2008; Serce *et al.,* 2010) TEAC and FRAP exhibited parallel results. Overall TAC values were 0.67 and 2.91 μmol TE g^{-1} fw for TEAC assay and 0.52 and 2.26 μmol TE g^{-1} fw for FRAP assay in green and red cultivars respectively. It is not surprising to observe higher antioxidant activity in red cultivars as anthocyanins have shown to be the predominant phenolic antioxidants in red fruits and vegetables (Stintzing *et al.,* 2002). Furthermore, outermost layers of red lettuce cultivars always accumulate more anthocyanin with the help of higher exposure of light.

Different from the previous studies, the experimental system used in our study afforded us to have precise measurement on distribution of TP and TAC by leaf positions within the heads of green and red cultivars that are grown in exactly same cultural and environmental condition side by side.

Effect of leaf position in TACY and chlorophyll content

Total anthocyanin (TACY) and chlorophyll (Chla and Chlb) profile of green and red lettuce cultivars were significantly affected by leaf positions (Table 3). Average TACY content of red lettuce cultivars was 5.82

Table 2. Total phenolic content (TP) and antioxidant capacity (TEAC and FRAP) of green and red lettuce cultivars affected by the leaf position. The values shown are mean of three replications. Means were compared by LSD ($p < 0.05$) within each column; means with the same letter do not differ significantly

Source	TP (µg GAE g^{-1} fw)			TEAC (µmol TE g^{-1} fw)			FRAP (µmol TE g^{-1} fw)		
	Red	Green	Mean	Red	Green	Mean	Red	Green	Mean
Leaf position									
Outer	845^{a}	350^{a}	488^{a}	5.63^{a}	0.95^{a}	3.29^{a}	4.27^{a}	0.63^{a}	2.45^{a}
Middle	297^{b}	225^{c}	261^{c}	1.60^{b}	0.58^{b}	1.09^{b}	1.62^{b}	0.53^{b}	1.07^{b}
Inner	195^{c}	245^{b}	329^{b}	1.49^{c}	0.48^{c}	0.99^{c}	0.91^{c}	0.38^{c}	0.65^{c}
$LSD_{0.05}$	3.59	4.70	2.88	0.09	0.01	0.04	0.05	0.01	0.03

Source	Outer	Middle	Inner	Mean	Outer	Middle	Inner	Mean	Outer	Middle	Inner	Mean
Leaf color												
Red	625.5^{a}	297.3^{a}	413.9	445.6^{a}	5.63^{a}	1.60^{a}	1.50^{a}	2.91^{a}	4.27^{a}	1.62^{a}	0.91^{a}	2.26^{a}
Green	350.5^{b}	224.6^{b}	244.9	273.3^{b}	0.95^{b}	0.58^{b}	0.50^{b}	0.67^{b}	0.63^{b}	0.53^{b}	0.38^{b}	0.52^{b}
$LSD_{0.05}$	165.84	33.17	ns	104.7	0.62	0.21	0.41	0.70	0.82	0.29	0.25	0.56
Cultivar												
Red												
Cherokee	$1{,}082.1^{a}$	313.9^{b}	204.6^{e}	533.5^{a}	6.70^{a}	2.20^{a}	0.70^{d}	3.20^{a}	6.16^{a}	2.30^{a}	1.08^{b}	3.18^{a}
Nation	868.7^{b}	365.5^{a}	145.3^{g}	459.8^{b}	6.37^{b}	1.40^{b}	1.70^{b}	3.14^{a}	2.66^{d}	1.13^{d}	0.57^{c}	1.45^{bc}
Paradai	740.6^{c}	237.3^{d}	199.5^{e}	392.5^{c}	5.13^{c}	1.40^{b}	1.10^{c}	2.55^{c}	4.44^{b}	1.38^{c}	0.50^{d}	2.11^{ab}
Versai	688.1^{d}	272.6^{c}	228.7^{c}	396.5^{c}	4.33^{d}	1.50^{b}	2.50^{a}	2.76^{b}	3.82^{c}	1.66^{b}	1.47^{a}	2.32^{ab}
Green												
Freckles	430.9^{e}	233.9^{d}	184.9^{f}	283.2^{e}	0.76^{f}	0.70^{c}	0.60^{e}	0.68^{e}	0.48^{g}	0.70^{e}	0.42^{e}	0.54^{c}
Fonseca	402.5^{f}	186.6^{e}	217.5^{d}	268.9^{f}	1.35^{e}	0.60^{c}	0.50^{f}	0.80^{d}	1.19^{f}	0.55^{ef}	0.37^{f}	0.70^{c}
Filipus	354.3^{g}	244.2^{d}	270.0^{b}	289.5^{d}	0.96^{f}	0.60^{c}	0.50^{f}	0.68^{e}	1.22^{e}	0.46^{f}	0.39^{ef}	0.69^{c}
Krizet	214.1^{h}	233.9^{d}	307.0^{a}	251.7^{g}	0.70^{f}	0.40^{d}	0.40^{f}	0.52^{f}	0.64^{g}	0.41^{f}	0.36^{f}	0.47^{c}
$LSD_{0.05}$	8.50	13.48	8.98	4.70	0.34	0.13	0.09	0.07	0.02	0.20	0.05	1.10
Proportion	136	73	92	100	184	61	55	100	176	77	46	100
Mean	488.0	261.0	329.4	359.4	3.3	1.1	1.0	1.8	2.6	1.1	0.6	1.4

µg g^{-1} fw. TACY content of outer, middle and inner leaves of red lettuce were 11.27, 3.20 and 2.98 µg g^{-1} fw respectively. 'Cherokee' and 'Nation' displayed the highest TACY content among the red cultivars. Mulabagal *et al.* (2010) compared *in vitro* biological activity of green and red lettuce and found out that the water extract of the red lettuce showed higher biological activity and anthocyanin content. The results of this study also indicated that the HPLC profile of the red lettuce extract showed only one anthocyanin and it was characterized as cyanidin-3-O-(6”-malonyl-β-glucopyranoside) which immediately converted to cyanidin-3-O-(6”-malonyl-β-glucopyranoside methyl ester) and cyanidin-3-O-β-glucopyranoside under laboratory conditions.

As expected, outer leaves of both green and red lettuce cultivars had higher Chla and Chlb content compared to other parts of lettuce. Average Chla content of green and red lettuce cultivars was 1.63 and 4.58 µg g^{-1} fw, average Chlb content of green and red lettuce cultivars was 3.91 and 4.38 µg g^{-1}, respectively. Freckles among the green cultivars and Cherokee among the red cultivars displayed the highest Chla and Chlb content.

Unlike anthocyanins, the molecules of chlorophyll are not soluble in the aqueous solution of cell sap. To maintain the amount of chlorophyll in their leaves, plants continuously synthesize it. The synthesis of chlorophyll in plants requires sunlight and warm temperatures. It is not surprising to observe higher pigment concentration and phytochemicals in outer leaves due to higher sun exposure. However, it is important to point out that the pigment concentration is directly influenced by phytochemical content of leaves; this is especially true for red lettuce cultivars.

Table 3. Total anthocyanin (TACY) and chlorophyll (Chla and Chlb) profile of green and red lettuce cultivars affected by leaf positions. The values shown are mean of three replications. Means were compared by LSD ($p < 0.05$) within each column; means with the same letter do not differ significantly

Source	TACY (µg g^{-1} fw)				Chl a (µgl g^{-1} fw)				Chl b (µg g^{-1} fw)			
	Red	Green	Mean		Red	Green	Mean		Red	Green	Mean	
Leaf position												
Outer	11.2^{a}	—	—		11.05^{a}	2.85^{a}	6.95^{a}		7.89^{a}	5.87^{a}	6.88^{a}	
Middle	3.20^{b}	—	—		2.36^{b}	0.52^{c}	1.44^{b}		3.12^{b}	2.49^{b}	2.80^{b}	
Inner	2.98^{c}	—	—		0.34	1.51^{b}	0.92^{c}		2.14^{c}	3.37^{b}	2.76^{b}	
LSD$_{0.05}$	0.18		—		0.78	0.50	0.45		0.82	0.95	0.61	
Source	**Outer**	**Middle**	**Inner**	**Mean**	**Outer**	**Middle**	**Inner**	**Mean**	**Outer**	**Middle**	**Inner**	**Mean**
Leaf color												
Red	11.27	3.20	2.98	5.82	11.05^{a}	2.36^{a}	0.34^{b}	4.58^{a}	7.89^{a}	3.1	2.1	4.38^{a}
Green	—	—	—	—	2.85^{b}	0.52^{b}	1.51^{a}	1.63^{b}	5.87^{b}	2.5	3.4	3.91^{a}
LSD$_{0.05}$	—	—	—	—	1.82	1.32	0.82	1.76	1.46	ns	ns	1.25
Red cultivars												
Cherokee	13.42^{a}	4.31^{a}	1.40^{d}	6.38^{a}	13.47^{ab}	5.71^{a}	0.26^{b}	6.48^{a}	8.93^{a}	5.34^{a}	1.91^{b}	5.39^{b}
Nation	12.73^{b}	2.80^{b}	3.30^{b}	6.28^{a}	10.76^{ab}	1.30^{b}	0.63^{b}	4.23^{bc}	6.73^{ab}	1.50^{c}	2.54^{b}	3.59^{cd}
Paradai	10.26^{c}	2.74^{bc}	2.27^{c}	5.09^{c}	11.14^{ab}	1.76^{b}	0.11^{b}	4.34^{b}	8.61^{a}	2.61^{bc}	1.93^{b}	4.39^{c}
Versai	8.67^{d}	2.94^{b}	4.93^{a}	5.52^{b}	8.81^{bc}	0.66^{b}	0.34^{b}	3.27^{d}	7.30^{ab}	3.02^{bc}	2.19^{b}	4.17^{c}
Green cultivars												
Freckles	—	—	—	—	6.41^{c}	1.01^{b}	3.26^{a}	3.56^{cd}	8.41^{a}	3.52^{b}	7.50^{a}	6.48^{a}
Fonseca	—	—	—	—	2.12^{d}	0.53^{b}	1.36^{b}	1.34^{e}	4.17^{b}	1.40^{c}	2.10^{b}	2.55^{e}
Filipus	—	—	—	—	1.64^{d}	0.38^{b}	0.31^{b}	0.78^{e}	6.23^{ab}	2.17^{bc}	2.49^{b}	3.63^{cd}
Krizet	—	—	—	—	1.24^{d}	0.16^{b}	1.12^{b}	0.84^{e}	4.67^{b}	2.86^{bc}	1.41^{b}	2.98^{de}
LSD$_{0.05}$	0.41	0.15	0.11	0.15	2.92	1.67	1.74	0.74	3.37	1.74	3.49	1.0
Proportion	184	61	55	100	224	46	30	100	166	68	67	100
Mean	11.3	3.2	2.6	3.6	0.9	3.1	6.9	1.4	6.9	2.8	2.8	4.1

—: assay not performed.

Despite the visual appearance red cultivars exhibited higher Chla and Chlb content. Li and Kubota (2009) also suggested that supplemental light may increase phytonutrient content of lettuce.

Effect of leaf position in TSS and color

Total soluble solids (TSS) and color readings (L*, a*, b*) of green and red lettuce cultivars were significantly affected by leaf positions (Table 4 and 5). Inner leaves displayed significantly higher TSS than middle and outer leaves in both red and green color lettuce. Average TSS content of green cultivars was significantly higher than red cultivars. TSS content of outer, middle and inner leaves of green lettuce was 2.60, 3.07 and 4.02%, respectively.

Leaf colors in relation to leaf position for each cultivar are presented in Table 5. As expected with natural coloration of lettuce, as a result of chlorophyll breakdown and anthocyanin accumulation L*, a*, and b* values dramatically changed. The *p*-values showed that all variables were significantly different among the leaf positions of lettuce. L* values, designating the lightness of the color, gradually decreased from inner to outer leaves. Similar patterns observed for a* values indicate color turn from green to red. Outer leaves of green cultivars displayed lower (more negative) a* values due to higher chlorophyll content with darker green color, while outer leaves of red cultivars exhibited higher a* values due to higher anthocyanin content with red color. b* values indicating color change from yellow to blue.

Results of our study are also explained by the maturity of leaves. Outer leaves are mature and fully ex-

Table 4. Total soluble solids (TSS) of green and red lettuce cultivars affected by leaf positions. The values shown are mean of three replications. Means were compared by LSD ($p < 0.05$) within each column; means with the same letter do not differ significantly

Source	TSS (%)			
	Red	**Green**	**Mean**	
Leaf position				
Outer	2.75^{c}	2.60^{c}	2.68^{c}	
Middle	2.94^{b}	3.07^{b}	3.01^{b}	
Inner	3.35^{a}	4.02^{a}	3.69^{a}	
Source	**Outer**	**Middle**	**Inner**	**Mean**
Leaf color				
Red	2.75	2.94	3.35^{b}	3.05^{b}
Green	2.60	3.07	4.02^{a}	3.28^{a}
$LSD_{0.05}$	ns	ns	0.32	0.19
Red cultivars				
Cherokee	2.85^{bc}	2.70^{c}	3.43^{c}	2.99^{bcd}
Nation	3.08^{ab}	3.08^{b}	3.30^{cd}	3.16^{bcd}
Paradai	3.00^{ab}	2.97^{b}	3.45^{c}	3.14^{bcd}
Versai	2.53^{de}	3.02^{b}	3.23^{cd}	2.93^{cd}
Green cultivars				
Freckles	2.92^{abc}	2.32^{d}	3.10^{d}	2.78^{d}
Fonseca	2.68^{cd}	3.32^{a}	4.03^{b}	3.34^{b}
Filipus	3.16^{a}	3.32^{a}	5.16^{a}	3.88^{a}
Krizet	2.40^{e}	3.34^{a}	4.00^{b}	3.25^{bc}
$LSD_{0.05}$	0.25	0.21	0.31	0.35
Proportion	89	95	116	100
Mean	2.8	3.0	3.7	3.2

panded leaves compared to inner leaves in the whole lettuce. As leaf completes its expansion and maturity the phytochemical accumulation may increase. Especially, in red lettuce cultivars anthocyanin sythesis dramatically increases by maturation. In addition, light exposure to outer leaves may result in incresed phytochemical accumulation. Also, outer leaves have direct contact to environmental conditions which makes them to produce more secondary metabolites than middle and inner parts.

Conclusions

In this study, we compared and quantified the phytonutrient content and antioxidant capacity of selected anthocyanin rich red lettuce and green lettuce cultivars. Green and red lettuce cultivars displayed different characteristics mainly due to the presence of anthocyanin in the red cultivars. More interestingly, distribution of TP, TACY and TAC greatly varied among the leaf positions; it was demonstrated that outer leaves have the highest phytonutrient content and antioxidant properties. However, to our knowledge, this is the first study to point the effect of leaf position on the antioxidant properties. Both researchers and consumers are likely to benefit from these results on distribution of phytochemicals and antioxidant capacity among the leaf positions of green and red lettuce cultivars that is reported here. The results have practical and scientific value, including breeding programs for cultivar development, sampling, human nutrition and pharmaceutical industry.

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Table 5. Color readings (L, a, b) of green and red lettuce cultivars affected by leaf positions. The values shown are mean of three replications. Means were compared by LSD ($p < 0.05$) within each column; means with the same letter do not differ significantly

Source	L*			a*			b*		
	Red	Green	Mean	Red	Green	Mean	Red	Green	Mean
Leaf position									
Outer	31.07^{c}	48.53^{c}	39.83^{c}	3.88^{a}	–21.75^{c}	–7.81^{a}	9.27^{c}	32.39^{c}	20.84^{c}
Middle	36.80^{b}	54.31^{b}	45.56^{b}	1.07^{a}	–19.50^{b}	–10.34^{b}	15.20^{b}	38.75^{b}	26.98^{b}
Inner	49.06^{a}	65.27^{a}	57.12^{a}	–3.53^{b}	–18.29^{a}	–10.92^{c}	25.22^{a}	42.58^{a}	33.90^{a}
$LSD_{0.05}$	3.55	1.71	1.56	3.07	0.91	1.29	3.30	1.77	1.38

Source	Outer	Middle	Inner	Mean	Outer	Middle	Inner	Mean	Outer	Middle	Inner	Mean
Leaf color												
Red	31.07^{b}	36.89^{b}	49.06^{b}	38.98^{b}	–19.50^{b}	–21.75^{b}	–18.29^{b}	–19.85^{b}	9.22^{b}	15.20^{b}	25.22^{b}	16.56^{b}
Green	48.58^{a}	54.31^{a}	65.17^{a}	56.02^{a}	3.88^{a}	1.03^{a}	–3.53^{a}	0.47^{a}	32.39^{a}	38.75^{a}	42.58^{a}	37.90^{a}
$LSD_{0.05}$	2.19	2.85	3.24	2.18	1.70	2.22	2.77	1.39	2.13	2.81	2.96	1.90
Red												
Cherokee	26.12^{c}	26.47^{e}	35.57^{e}	29.4^{d}	6.42^{a}	8.66^{a}	6.95^{a}	7.3^{a}	3.10^{e}	4.03^{f}	12.79^{e}	6.6^{f}
Nation	31.64^{b}	42.08^{c}	59.14^{bc}	44.3^{b}	8.03^{a}	3.10^{b}	–4.93^{b}	2.1^{b}	11.45^{cd}	18.92^{d}	27.26^{d}	19.2^{d}
Paradai	34.69^{b}	42.96^{c}	55.23^{c}	44.3^{b}	–0.39^{b}	–7.06^{d}	–10.90^{c}	–6.1^{d}	14.14^{c}	23.40^{c}	34.37^{c}	24.0^{c}
Versai	31.85^{b}	35.72^{d}	46.31^{d}	38.0^{c}	1.46^{b}	–0.41^{c}	–5.27^{b}	–1.4^{c}	8.42^{d}	14.47^{e}	26.46^{d}	16.5^{e}
Green												
Freckles	48.48^{a}	54.12^{ab}	66.05^{a}	56.2^{a}	–20.04^{c}	–22.50^{e}	–16.12^{d}	–19.6^{e}	32.41^{ab}	41.53^{a}	45.60^{a}	39.8^{a}
Fonseca	47.57^{a}	55.42^{ab}	65.75^{a}	56.2^{a}	–20.15^{c}	–22.67^{e}	–19.11^{d}	–20.6^{e}	32.57^{ab}	40.58^{a}	39.48^{b}	37.5^{b}
Filipus	47.28^{a}	51.13^{b}	65.55^{a}	54.7^{a}	–18.46^{c}	–22.08^{e}	–19.52^{d}	–20.0^{e}	29.84^{b}	37.60^{ab}	47.05^{a}	38.2^{ab}
Krizet	51.01^{a}	56.57^{a}	63.34^{ab}	57^{a}	–19.39^{c}	–19.78^{e}	–18.44^{d}	–19.2^{e}	34.76^{a}	35.31^{b}	38.20^{bc}	36.1^{b}
$LSD_{0.05}$	4.13	4.59	4.58	2.55	2.97	3.37	4.48	2.10	3.69	4.14	3.97	2.26
Proportion	84	96	120	100	81	107	113	100	76	99	124	100
Mean	39.8	45.6	57.1	47.5	–7.8	–10.3	–10.9	–9.7	20.8	27	33.9	27.2

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