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Antioxidant Capacity and Phenolic Content of Spinach As Affected by Genetics and Maturation

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Spinach leaves harvested at three maturity stages from eight commercial cultivars (CC) and eight advanced breeding lines (ABL) were evaluated for oxygen radical absorbing capacity (ORAC), total phenolics, and flavonoid composition and content. ABL had higher levels of total phenolics, total flavonoids, and ORAC than CC. Midmaturity spinach leaves had higher levels of total phenolics, total flavonoids, and antioxidant capacity than immature and mature leaves. The contents of individual flavonoids varied in response to maturation, with the predominant glucuronated flavones decreasing and patuletin and spinacetin derivatives increasing. Both total phenolics and total flavonoids correlated well with ORAC (r_{xy} = 0.78 and 0.81, respectively) demonstrating that flavonoids were major contributors to antioxidant capacity. Our results indicate that spinach genotypes should be harvested at the midmaturity stage for consumers to benefit from elevated levels of health promoting flavonoids present in the leaves. Additionally, plant breeders can select for increased phenolic content to increase antioxidant capacity of spinach genotypes.

KEYWORDS: Antioxidant capacity; flavonoids; genotype; maturity; spinach

INTRODUCTION

Flavonoids are a class of secondary plant phenolics that are thought to exert beneficial health effects through their antioxidant and chelating properties. In addition to antioxidant function, flavonoids may also modulate cell signaling pathways and could have marked effects on cellular function by altering protein and lipid phosphorylation and modulating gene expression (*1*). Spinach (*Spinacia oleracea*) has a notable flavonoid content (>1000 mg/kg) (*2*, *3*) compared to other flavonoid-rich vegetables such as broccoli (*4*) and red onion (*5*). Spinach is devoid of flavonoids normally present in most fruits and vegetables such as quercetin (3,5,7,3′,4′-pentahydroxyflavone), kaempferol (3,5,7,4′-tetrahydroxyflavone), or myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) but contains unique flavonoid compounds including glucuronides and acylated di- and triglycosides of methylated and methylenedioxyderivatives of 6-oxygenated flavonols (*6*−*9*) (**Figure 1**). Spinach also contains many hydroxycinnamic acids: feruoylglucose, trans and cis isomers of *p*-coumaric acid, and *meso*-tartarate derivatives of *p*-coumaric acid in addition to flavonoids (*10*, *11*).

Because of an abundance of phenolic compounds, spinach ranks high among vegetables in terms of antioxidant capacity (*12*, *13*), suggesting that spinach consumption may afford protection against oxidative stress mitigated by free-radical species. Oxidative stress is associated with numerous chronic diseases, and it is thought that increased dietary intake of antioxidants may be important in disease prevention (*14*). Spinach phenolic compounds exhibit a wide range of biological effects including antioxidative (*11*, *15*), anti-inflammatory (*16*), antiproliferative (*17*), anticarcinogenic (*18*), and antimutagenic (*9*) properties. Spinach extracts may play an important role in chemoprevention, central nervous system protection, and anti-aging functions in mammals (*19*). Many potential health benefits associated with spinach are thought to be related to unique flavonoids present in the leaves.

Fruit and vegetable phenolic content, including flavonoids, can be influenced by several factors, including genetics, cultivation practices, environmental, growing conditions, maturation, storage, and processing. Spinach phenolic content and antioxidant capacity are influenced by genetics (*3*), growing season (*3*), minimal processing, domestic cooking (*2*), and frozen storage (*20*, *21*). However, the effect of leaf maturation on flavonoid content and antioxidant capacity of spinach is unknown.

This study was undertaken to determine how leaves from eight commercial cultivars (CC) and eight advanced breeding lines (ABL) of spinach harvested at three different maturity stages varied in total phenolics, flavonoid composition/content, and antioxidant capacity.

MATERIALS AND METHODS

Leaf Sampling. Spinach plants representing eight CC and eight ABL were cultivated in fall 2003 at the University of Arkansas, Vegetable

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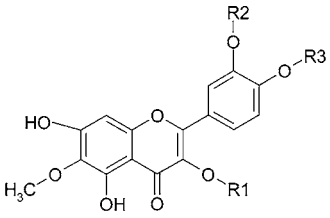
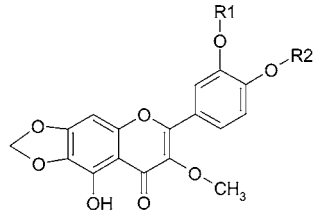


Compound 1 "Patuletin" R1 = β-D-glucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = H; R3 = H
Compound 2 "Spinacetin" R1 = β-D-glucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = CH_3; R3 = H
Compound 3 "Patuletin" R1 = β-D-2" feruloylglucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = H; R3 = H
Compound 4 "Spinacetin" R1 = β-D-2" *p*-coumaroylglucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = CH_3; R3 = H
Compound 5 "Spinacetin" R1 = β-D-2"feruloylglucosyl (1→6) β-D-apiosyl (1→2) β-D-glucoside; R2 = CH_3; R3 = H
Compound 6 "Spinacetin" R1 = β-D-glucosyl (1→6) β-D-glucoside; R2 = CH_3; R3 = H
Compound 7 "Jaceidin" R1= CH_3; R2 = CH_3; R3 = glucuronic acid



Compound 8 R1 = H; R2 = glucuronic acid
Compound 9 R1 = CH_3; R2 = glucuronic acid

Figure 1. Chemical structures of spinach flavonoids.

Substation, Kibler, AR. The field plot plan was a randomized complete block with four replications. The plants were grown under identical growing conditions and received uniform fertility, irrigation, and herbicide treatments. The leaves were collected on the same day within 2 h from 10 randomly selected plants of each genotype at three maturity stages: immature (small leaves obtained from the top of the plant, leaf width < 3.81 cm), midmature (medium size leaves obtained from the middle of the plant, leaf width 3.81−6.35 cm), and mature (large leaves obtained from the base of the plant, leaf width > 6.35 cm). Samples were frozen immediately after harvest, lyophilized, placed in brown vials, sealed, and stored at −20 °C.

Chemical Analyses. *Chemicals.* Fluorescein, Folin-Ciocalteu reagent, ethylenediamine tetraacetic acid (EDTA), gallic acid, and citric acid were purchased from Sigma Chemical Co. (St. Louis, MO). 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (trolox) was obtained from Aldrich (Milwaukee, WI), and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA, Inc. (Richmond, VA). HPLC grade methanol was obtained from VWR (West Chester, PA).

Sample Preparation for Determination of Total Phenolics, Flavonoids, and Oxygen Radical Absorbing Capacity (ORAC). A modified procedure of Gil et al. (*2*) was used for extraction of phenolics from spinach powder. Spinach powder (20 g) was homogenized with 80 mL MeOH:H_2O (5:95) containing 0.5 g/L citric acid and 0.5 g/L EDTA using a Euro Turrax model T18 tissuemizer (Tekmar-Dohrman Corp., Mason, OH). Extracts were filtered through Miracloth (CalBiochem, La Jolla, CA) and were stored at −20 °C until analysis. Frozen extracts were thawed and diluted with phosphate buffer and deionized water, respectively, prior to ORAC and total phenolic assays.

Total Phenolics Assay. Total soluble phenolics in the methanol/water/citric acid/EDTA extracts were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (*22*) using gallic acid as a standard. Results were expressed as mg of gallic acid equivalents per g of dry weight.

ORAC Assay. The automated oxygen radical absorbing capacity (ORAC) assay was carried out with a Fluostar Optima microplate reader (BMG Labtechnologies, Durham, NC) as described by Prior et al. (*23*). Spinach extracts were diluted 200-fold or more with phosphate buffer (75 mM, pH 7.0). The diluted extract (40 μL) was added to each well in clear 48-well Falcon plates. Phosphate buffer was used as a blank and trolox (40 μL) was used as the standards (6.25, 12.5, 25, and 50 μM). The Fluostar Optima instrument, equipped with two automated injectors, was then programmed to add 400 μL of fluorescein (0.11 μM) followed by 150 μL of AAPH (31.6 mM) to each well. Fluorescence readings (excitation 485 nm, emission 520 nm) were recorded immediately after the addition of fluorescein, immediately after the addition of AAPH, and every 192 s thereafter for 90 min to reach a 95% loss of fluorescence. Final fluorescence measurements were expressed relative to the initial reading. Results were calculated on the basis of differences in areas under the fluorescein decay curve between the blank, samples, and trolox standards. A standard curve was obtained by plotting the four concentrations of trolox standards against the net area under the curve of each standard. Final ORAC values were calculated using the regression equation between trolox concentration and area under the curve and were expressed as μmoles of trolox equivalents (TE) per g of dry weight (DW).

Flavonoid Analysis. Flavonoids were analyzed by HPLC as described by Gil et al. (*2*). Extracts were filtered through Whatman no. 4 filter paper and were then passed through a 0.45-μm filter prior to HPLC analysis. The samples (40 μL) were injected into a Waters HPLC system (Milford, CT) equipped with a LiCrochart column (RP-18, 12.5 × 0.4 cm, 5-μm particle size) (Merck, Darmstadt, Germany). Elution was by water/formic acid (19:1, v:v) (A) and HPLC-grade MeOH (B) as the mobile phases, on a gradient starting with 10% B in A to reach 40% B at 30 min and 80% B at 40 min. The flow rate was 1 mL/min, and absorption at 280 and 350 nm was recorded using a Waters 996 photodiode-array detector. Peaks were identified and quantified using external standards previously isolated from spinach (*2*, *6*−*8*). The concentrations of individual flavonoids were summed and expressed as total flavonoid content (mg/g DW).

Statistical Analyses. Data represent the mean of four replicate analyses. Analysis of variance was performed using JMP software (*24*) to determine effects of genotype, maturity, and genotype × maturity interaction on all dependent variables. Mean values were compared using Student's *t* test at 5% level. Correlation analysis between ORAC values and total phenolics and flavonoids was also performed using JMP software.

RESULTS

Genotypic and Maturity Effects on Total Phenolics, Flavonoids, and ORAC. Analysis of variance showed that main effects for all variables were significant (**Table 1**). Variation in ORAC, total phenolics, and total flavonoids between maturity stages was much greater than that between genotypes, indicating that maturity plays a more important role than genotype in influencing ORAC, total phenolics, and total flavonoids in spinach. Significant main effects for genotype and genotype × maturity for ORAC, total phenolics, and total flavonoids demonstrated that different genotypes varied in their capacity to synthesize phenolics at different maturity stages.

Total phenolic content of 16 spinach genotypes at three maturity stages is shown in **Figure 2**. Over all maturity stages, ABL 91-227 had the highest level of total phenolics, whereas the CC St. Helen and Wintergreen had the lowest levels. The total phenolic levels in immature leaves ranged from 12.1 to 31.4 mg/g DW, reflecting a 2.3-fold difference among genotypes, whereas 2.6 and 2.3-fold differences in total phenolics among genotypes were found in both midmature (17.6−46.6 mg/g DW) and mature leaves (15.0−35.2 mg/g DW). Nine genotypes had higher levels of total phenolics at the midmaturity stage compared to the immature and mature stages, whereas three genotypes had similar levels of total phenolics over the three maturity stages. Spinach leaves harvested at the immature and mature stages generally had similar levels of total phenolics, except for five genotypes, which had higher levels at the mature stage and one genotype that had higher levels at the immature

Table 1. Analysis of Variance for Oxygen Radical Absorbing Capacity (ORAC), Total Phenolics, and Total Flavonoids

	ORAC				total phenolics			total flavonoids		
source of variation	DF[a]	MS[b]	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
genotype (*G*)	15	26620.3	116.0	<0.0001	321.0	29.1	<0.0001	41.5	838.3	<0.0001
maturity (*M*)	2	273372.7	1191. 1	<0.0001	1179. 2	91.2	<0.0001	68.6	1696. 4	<0.0001
$G \times M$	30	8389.4	36.5	<0.0001	43.6	3.8	<0.0001	21.2	410.8	<0.0001

[a] Degrees of freedom. [b] Mean square.

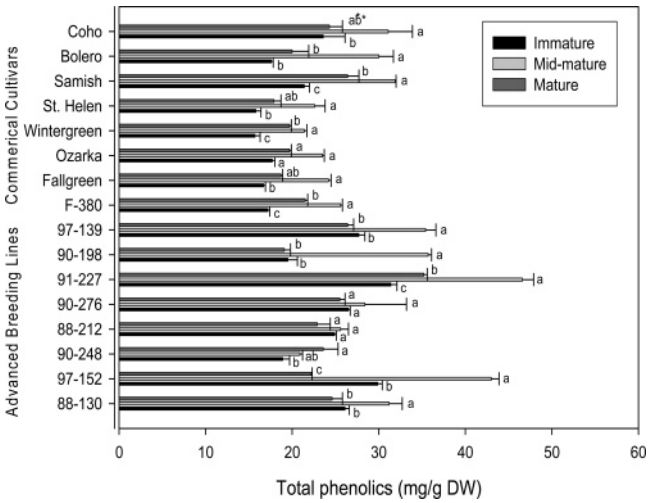


Figure 2. Total phenolic content of spinach genotypes at three maturity stages. *Mean values ± SEM ($n = 4$) represented by bars within each genotype with similar letters are not significantly different (Student's *t* test, $P \geq 0.05$).

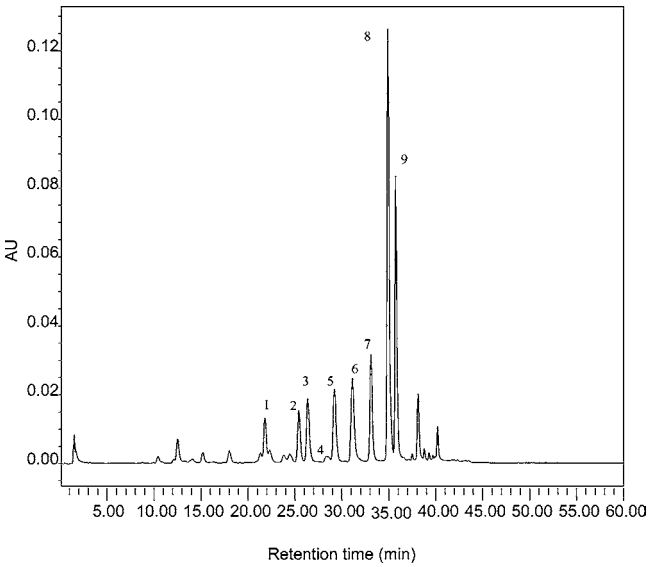


Figure 3. HPLC chromatogram (360 nm) of spinach flavonoids. See **Figure 1** for flavonoid identification.

stage. When total phenolics for all maturity stages were averaged for each genotype, ABL had higher levels of total phenolics than CC.

A typical HPLC chromatogram of spinach flavonoids is shown in **Figure 3**. The eight predominant flavonoids present (excluding peak 4) in the extracts that have previously been identified (*25*−*27*) were summed and expressed as total flavonoids. The total flavonoid content of spinach genotypes at three maturity stages is shown in **Figure 4**. Over all maturity stages, ABL 91-227 had the highest level of total flavonoids, while ABL 88-212 had the lowest level. Total flavonoid content

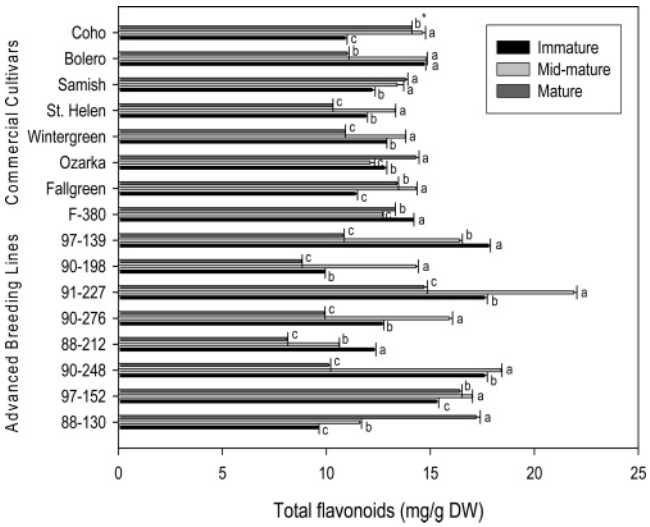


Figure 4. Total flavonoid content of spinach genotypes at three maturity stages. *Mean values ± SEM ($n = 4$) represented by bars within each genotype with similar letters are not significantly different (Student's *t* test, $P \geq 0.05$).

of immature leaves ranged from 8.7 to 17.8 mg/g DW, reflecting a 2.0-fold difference, whereas total flavonoid contents of midmature and mature spinach leaves ranged from 11.6 to 21.9 and from 6.6 to 16.4 mg/g DW, respectively. Nine genotypes had the highest levels of total flavonoids at the midmature stage, whereas three genotypes had highest levels at the immature stage, and two genotypes had the highest levels at the mature stage. Ten genotypes had higher levels of total flavonoids at the immature stage compared to the mature stage, while six genotypes had higher levels at the mature stage compared to the immature stage. A moderate correlation was obtained between total flavonoids and total phenolics ($r_{xy} = 0.64$, $P \leq 0.01$) for all genotypes, suggesting that in addition to flavonoids other phenolics or nonphenolic compounds with reducing capacity were present in the extracts. ABL contained higher mean values of total flavonoids than CC at the three maturity stages, which was consistent with the total phenolic results.

The oxygen radical absorbing capacity of 16 spinach genotypes at three maturity stages is shown in **Figure 5**. Over all maturity stages, ABL 91-227 had the highest ORAC value, while the CC Samish had the lowest value. ORAC values of immature leaves from the genotypes ranged from 104.2 to 252.8 μmoles TE/g DW, reflecting a 2.4-fold difference, whereas ORAC values of midmature and mature spinach leaves from the genotypes ranged from 168.4 to 461.7 and from 119.0 to 288.8 μmol TE/g DW, respectively, reflecting 2.7- and 2.4-fold differences. In all genotypes (excluding 88-212), leaves harvested at the midmature stage had the highest ORAC values. Eight genotypes had higher ORAC values at the mature stage compared to the immature stage, while four genotypes had higher ORAC values at the immature stage compared to the mature stage.

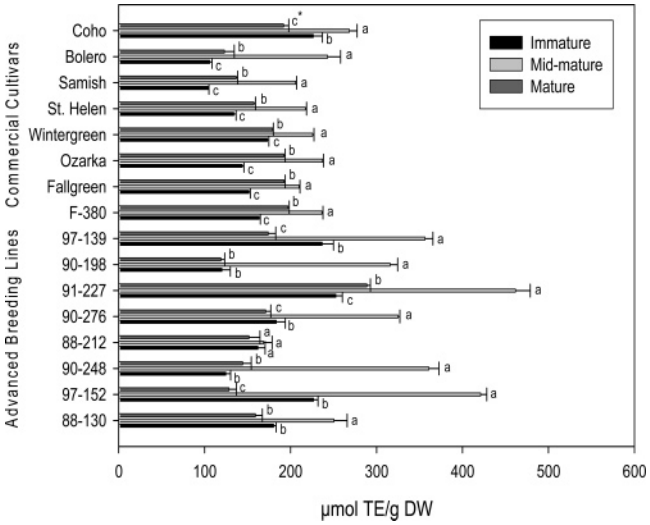


Figure 5. ORAC values of spinach genotypes at three maturity stages. *Mean values ± SEM ($n = 4$) represented by bars within each genotype with similar letters are not significantly different (Student's t test, $P \geq 0.05$).

A large variation in content of individual flavonoids was observed among spinach genotypes harvested at three maturity stages (**Table 2**). A trend of mature > midmature > immature was observed for compounds **1**, **2**, **5**, and **6** for both ABL and CC. For compound **3**, midmaturity leaves from ABL had the highest content followed by mature and immature leaves, whereas in CC, mature leaves had the highest content followed by mid- and immature leaves. Midmaturity leaves from ABL had the highest content of compound **6** followed by mature and immature leaves, whereas in CC mature leaves had the highest content followed by mid- and immature leaves. For compound **7**, midmature leaves from ABL had the highest content followed by mature and immature leaves, whereas in CC, mature leaves had the highest content followed by mid- and immature leaves. Immature leaves from ABL had the highest content of compound **8** followed by mid and mature leaves, whereas in CC mature leaves had the highest content followed by immature and midmature leaves. Immature leaves from ABL and CC had the highest content of compound **9** followed by midmature leaves, which had much higher levels of the compound than mature leaves. Among all genotypes, a trend of mature > midmature > immature was observed for compounds **1**, **2**, **5**, and **6**. Midmature leaves had the highest content of compound **3** followed by mature and immature leaves, while mid and mature leaves had higher contents of compound **7** than immature leaves. A trend of immature > midmature > mature was observed for compound **9**, while immature leaves contained higher levels of compound **8** than midmature and mature leaves, which had similar levels.

The mean values of total phenolics, total flavonoids, and ORAC values of spinach genotypes at three maturity stages are presented in **Table 3**. In ABL, total phenolics increased 30.0% from immature to midmature stage and then decreased 25.2% from the midmature to mature stage. In CC, total phenolics increased 44.5% from the immature to midmature stage and then decreased 20.2% from the midmature to mature stage. For all genotypes, total phenolics increased 36.1% from the immature stage to the midmature stage and then decreased 23.2% from the midmature to mature stage. ABL had higher levels of total phenolics than CC over all maturity stages In ABL, total flavonoids increased 12.1% from immature to midmature stage and then decreased 24.1% from the midmature to mature stage, whereas in CC total flavonoids increased 7.9% from the immature to midmature stage and then decreased 6.6% from midmature to mature stage. For all genotypes, total flavonoids increased 9.7% from immature to midmature stage and then decreased 16.3% from midmature to mature stage. ABL had higher levels of total flavonoids than CC at immature and midmature stages, but the ABL and CC had similar levels of total flavonoids at the mature stage. In ABL, ORAC increased 78.9% from immature to midmature stage and then decreased 49.8% from midmature to mature stage. In CC, ORAC increased 53.4% from immature to midmature stage and then decreased 26.3% from midmature to mature stage. For all genotypes, ORAC increased 67.5% from immature to midmature stage and then decreased 40.2% from midmature to mature stage. ABL had higher ORAC values than CC at immature and midmature stages, but the ABL and CC had similar ORAC values at the mature stage.

The correlation coefficients between ORAC, total phenolics, and individual and total flavonoids in spinach genotypes over all maturity stages are shown in **Table 4**. A moderate correlation was observed between ORAC values and total phenolics over all maturity stages ($r_{xy} = 0.78$), indicating that variation in phenolics formed in response to maturation affected the antioxidant activities of spinach leaves. A similar correlation was observed between ORAC values and total flavonoids ($r_{xy} = 0.81$). Peaks 1, 2, and 5 all correlated moderately with ORAC ($r_{xy} = 0.85$, 0.80, and 0.78, respectively) with significance at P

Table 2. Individual Flavonoid Content of Spinach Genotypes at Three Maturity Stages[a]

maturity stage	1	2	3[b]	5	6[c]	7	8	9
				Advanced Breeding Lines				
immature	0.21 ± 0.03c[d]	0.94 ± 0.04c	1.13 ± 0.08c	1.13 ± 0.03c	0.82 ± 0.03c	0.48 ± 0.03c	2.01 ± 0.06a	7.38 ± 0.13a
midmature	0.50 ± 0.04b	1.40 ± 0.06b	1.43 ± 0.05a	1.63 ± 0.06b	1.23 ± 0.04a	0.69 ± 0.03a	1.88 ± 0.04b	7.03 ± 0.13b
mature	0.60 ± 0.06a	1.53 ± 0.12a	1.28 ± 0.05b	1.79 ± 0.06a	1.10 ± 0.03b	0.61 ± 0.03b	1.23 ± 0.04c	3.70 ± 0.08c
				Commercial Cultivars				
immature	0.23 ± 0.03c	0.72 ± 0.03c	0.71 ± 0.05c	0.76 ± 0.03c	0.55 ± 0.02c	0.43 ± 0.02c	1.64 ± 0.03b	7.60 ± 0.16a
midmature	0.34 ± 0.02b	0.92 ± 0.02b	0.99 ± 0.03b	1.17 ± 0.03b	0.98 ± 0.04b	0.69 ± 0.02b	1.34 ± 0.04c	7.18 ± 0.28b
mature	0.39 ± 0.04a	1.28 ± 0.04a	1.02 ± 0.05a	1.31 ± 0.04a	1.35 ± 0.05a	0.76 ± 0.04a	1.93 ± 0.07a	4.63 ± 0.18c
				All Genotypes				
immature	0.22 ± 0.03c	0.83 ±0.04c	0.92 ±0.07c	0.95 ± 0.03c	0.68 ± 0.02c	0.46 ± 0.02b	1.83 ± 0.05a	7.49 ± 0.15a
midmature	0.45 ± 0.03b	1.19 ±0.04b	1.21 ±0.04a	1.43 ± 0.05b	1.12 ± 0.04b	0.70 ± 0.02a	1.59 ± 0.04b	6.91 ± 0.22b
mature	0.49 ± 0.05a	1.40 ± 0.09a	1.15 ± 0.05b	1.55 ± 0.05a	1.22 ± 0.04a	0.68 ± 0.03a	1.58 ± 0.05b	4.16 ± 0.14c

[a] Data expressed as milligrams per gram dry weight represents mean values ± SEM ($n = 8$ for ABL and CC, and $n = 16$ for all genotypes) of 16 spinach genotypes. See **Figure 3** for compound identification. [b] Data quantified as compound **1**. [c] Data quantified as compound **5**. [d] Data within columns under each heading with similar letters are not significantly different (Student's t test, $P \geq 0.05$).

Table 3. Mean Values of Total Phenolics, Total Flavonoids, and ORAC Values of Spinach Genotypes at Three Maturity Stages

maturity stage	total phenolics[a]	total flavonoids[b]	ORAC[c]
	Advanced Breeding Lines		
immature	25.6 ± 0.8b[d,e]	14.1 ± 0.6b[e]	185.9 ± 8.8b[e]
midmature	33.3 ± 1.6a[e]	15.8 ± 0.6a[e]	332.4 ± 15.9a[e]
mature	24.9 ± 0.8b[e]	12.0 ± 0.6c	167.0 ± 9.2b
	Commercial Cultivars		
immature	18.2 ± 0.5c	12.6 ± 0.2b	150.3 ± 6.8c
midmature	26.3 ± 0.8a	13.6 ± 0.2a	230.5 ± 3.9a
mature	21.0 ± 0.6b	12.7 ± 0.3b	169.8 ± 4.9b
	All Genotypes		
immature	21.9 ± 0.7b	13.4 ± 0.3b	168.1 ± 5.9b
midmature	29.8 ± 0.9a	14.7 ± 0.3a	281.5 ± 10.4a
mature	22.9 ± 0.6b	12.3 ± 0.3c	168.4 ± 5.2b

[a] Milligrams of gallic acid equivalents per gram dry weight ± SEM. [b] Milligrams of flavonoids per gram dry weight ± SEM. [c] Micromoles of trolox equivalents per gram dry weight ± SEM. [d] Data within columns under each heading with similar letters are not significantly different (Student's *t* test, $P \geq 0.05$, $n = 8$ for ABL and CC, and $n = 16$ for all genotypes). [e] Indicates significant difference between advanced breeding lines and commercial cultivars (Student's *t* test, $P \leq 0.05$).

Table 4. Pearson's Correlation Coefficients[a] for ORAC with Total Phenolics, and Individual and Total Flavonoids in Spinach Genotypes over All Maturity Stages

compound	r_{xy}
total phenolics	0.78***
total flavonoids	0.81***
peak 1	0.85***
peak 2	0.80***
peak 3	0.74**
peak 5	0.78***
peak 6	0.72**
peak 7	0.55*
peak 8	0.66**
peak 9	0.20 ns

[a] *, **, ***, and ns designate significance at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and nonsignificance, respectively ($n = 16$).

≤ 0.001. Less linear relationships were observed between ORAC and compounds **3**, **6**, and **8** ($P \leq 0.01$) as well as compound **7** ($P \leq 0.05$). Peak 9, the predominant flavonoid in spinach, did not correlate significantly with ORAC.

DISCUSSION

Both genotype and maturity had marked effects on total phenolics, total flavonoids, and ORAC in spinach. The significant effect of genotype was consistent with a previous study, where genotype and growing season significantly influenced ORAC, total phenolic, and flavonoid content of spinach (*3*). Spinach ABL had higher levels of total phenolics and total flavonoids than CC, which also confirmed our previous results (*3*). Advanced selections from the breeding program have undergone repeated field selections for resistance to white rust and downy mildew (*25*), and it appears that the selection process for disease resistance has increased phenolic levels in the breeding materials. In addition to enhanced antioxidant capacity, it is possible that elevated levels of phenolics in the germplasm may confer disease protection through phytoalexic activity.

Midmature leaves had much higher levels of total phenolics, total flavonoids, and ORAC values than immature and mature leaves. These results indicate that the synthesis of phenolics occurred readily from the immature to midmature stage and then may have slowed or ceased as leaves expanded to the mature stage. If the leaves accumulated solids during expansion and phenolic synthesis was arrested, then a reduction in phenolics would occur because of a dilution effect. The effect of maturation on flavonoids appears to vary among fruits and vegetables. Flavonoid levels in peppers (*26*, *27*), cabbage (*28*), and colored potato tubers (*29*) decreased in response to maturation, flavonoid levels in strawberries were unaffected by maturation (*30*), while flavonoids in broad beans increased as pods matured (*31*).

Although the total flavonoid content was highest at the midmature stage, the levels of individual flavonoids varied with maturation. Several of the minor flavonoid constituents (compounds **1**, **2**, and **5**) increased during maturation, whereas the predominant flavonoid (compound **9**) decreased. A major loss of compound **9** during maturation greatly affected total flavonoid content of the genotypes. The compound accounted for 56% of total flavonoids in immature leaves but only 34% of total flavonoids in mature leaves. These results suggest that the ability of leaves to synthesize glucuronated flavones was retarded during maturation, and phenolic precursors were possibly shunted to synthesis of patuletin and spinacetin derivatives.

The moderate correlations observed between ORAC and total phenolics ($r_{xy} = 0.78$) and total flavonoids ($r_{xy} = 0.81$) in the genotypes over all maturity stages indicated that additional phenolic compounds or nonphenolic compounds with reducing capacity contributed to ORAC. Other phenolic compounds in spinach that may contribute to antioxidant capacity include feruloylglucose, trans and cis isomers of *p*-coumaric acid, and *meso*-tartrate derivatives of *p*-coumaric acid (*10*, *11*).

Changes in flavonoid composition during maturation most likely impacted the ORAC values. The concentrations of flavonoids (compounds **1**, **3**, and **5**) with significant activity against the 2,2-diphenyl-1-picrylhydrazyl radical (*2*) were highest in midmature or mature leaves. The two patuletin derivatives (compounds **1** and **3**) have a 3′,4′-dihydroxyl grouping on the B ring, which enhances radical scavenging capacity, whereas the spinacetin derivative (compound **5**) has a methoxy group at C3 which reduces radical scavenging capacity. Although compound **3** is reported to have lower antioxidant activity (0.62 trolox equivalents) than compound **1** (0.94 trolox equivalents) (*2*), the concentration of compound **3** in spinach leaves was much higher than compound **1**. Compound **5** is the only spinacetin derivative with significant free-radical scavenging activity (0.28 trolox equivalents) (*2*). The compound was highest in mature leaves in both ABL and CC. Flavonoids with no or minor activity against free radicals (jaceidin, other spinacetin derivatives, and glucuronated flavones) varied in concentration in response to maturation. As previously reported (*2*), jaceidin and the glucuronated flavones show no radical scavenging capacity because of the glucuronide moieties being attached at the 4′-position. Compound **6**, which is also a spinacetin derivative, was the highest in midmature leaves for ABL but highest in mature leaves of CC. Jaceidin was highest in midmature leaves of both ABL and CC, while compound **9**, a glucuronated flavone, was highest in immature leaves. These results suggest that enzymes involved in flavonoid biosynthesis are influenced by maturity. The highest ORAC values observed in midmature leaves, which had the highest levels of total flavonoids, reflected decreased levels of glucuronated flavones and increased levels of patuletin and spinacetin derivatives. On the basis of reported TEAC values for individual flavonoids (*2*), one would expect that mature leaves would have the highest ORAC values since the leaves had the highest levels of spinacetin and patuletin derivatives and the lowest levels of

glucuronated flavones. This discrepancy could be due to many factors including synergistic/antagonistic interactions among flavonoids, different reactivities of the individual flavonoids in the TEAC and ORAC assays, or the contribution of other phenolic compounds to antioxidant capacity.

CONCLUSIONS

Spinach leaves harvested at the midmature stage had much higher levels of total phenolics, total flavonoids, and antioxidant capacity than immature and mature leaves. Our results indicate that maturity stage should be considered for consumers to benefit from the antioxidant-rich flavonoids in spinach. Since immature "baby" leaves are commonly used in packaged salad mixes, fresh-cut processors may want to consider the use of midmature leaves to boost the flavonoid content and antioxidant capacity of their products.

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