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ARTICLE

Relationship between Fresh-Packaged Spinach Leaves Exposed to Continuous Light or Dark and Bioactive Contents: Effects of Cultivar, Leaf Size, and Storage Duration

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Current retail marketing conditions allow produce to receive artificial light 24 h per day during its displayed shelf life. Essential human-health vitamins [ascorbic acid (vit C), folate (vit B_9), phylloquinone (vit K_1), α-tocopherol (vit E), and the carotenoids lutein, violaxanthin, zeaxanthin, and β-carotene (provit A)] also are essential for photosynthesis and are biosynthesized in plants by light conditions even under chilling temperatures. Spinach leaves, notably abundant in the aforementioned human-health compounds, were harvested from flat-leaf 'Lazio' and crinkle-leafed 'Samish' cultivars at peak whole-plant maturity as baby (top- and midcanopy) and larger (lower-canopy) leaves. Leaves were placed as a single layer in commercial, clear-polymer retail boxes and stored at 4 °C for up to 9 days under continuous light (26.9 $\mu mol \cdot m^2 \cdot s$) or dark. Top-canopy, baby-leaf spinach generally had higher concentrations of all bioactive compounds, on a dry weight basis, with the exception of carotenoids, than bottom-canopy leaves. All leaves stored under continuous light generally had higher levels of all bioactive compounds, except β-carotene and violaxanthin, and were more prone to wilting, especially the flat-leafed cultivar. All leaves stored under continuous darkness had declining or unchanged levels of the aforementioned bioactive compounds. Findings from this study revealed that spinach leaves exposed to simulated retail continuous light at 4 °C, in clear plastic containers, were overall more nutritionally dense (enriched) than leaves exposed to continuous darkness.

KEYWORDS: *Spinacia oleracea*; antioxidants; ascorbic acid; β-carotene; folate; lutein; 5-methyltetrahydrofolate; tocopherol; phylloquinone; postharvest storage; violaxanthin; zeaxanthin

INTRODUCTION

Fresh spinach (*Spinacia oleracea* L.), arguably one of the most nutritionally complete vegetables commonly consumed, provides 20% or more of the recommended dietary intake of ascorbic acid (vitamin C), carotenoids (provitamin A), folate (vitamin B_9), phylloquinone (vitamin K), and α-tocopherol (vitamin E) (*1*). The role of vitamins C (*2*), K (*3*), and E (*4*) and carotenoids (*5*) in the prevention of chronic diseases has been well documented. The combined role in humans and plants of vitamins C (*6*), K (*7*), and E (*8*) and cartoenoids (*9*) are as antioxidants, whereas vitamin K acts as a redox cofactor (*10*, *11*) and folate (*12*, *13*) as a methyl donor. Each of these vitamins is found in the chloroplast of plants. The light reaction of photosynthesis, being temperature independent, can occur at 4 °C when light intensity is sufficient (*14*). Retail grocery markets currently display packaged spinach at 4 °C in light-transmissible polymers, which are exposed to 24 h continuous artificial light if the package is located at the front of the display case or near continuous darkness if the package is located at the back of the case. It is therefore likely that spinach can be subjected to hours, if not days, of continuous light, affecting the photosynthetic system-associated carotenoids and vitamins C, B_9, E, and K contents and overall stress-associated antioxidant capacity (*15*).

The purpose of this study was to determine how continuous light exposure of commercial flat-leaf 'Lazio' and crinkle-leaf 'Samish' spinach types stored in clear, retail packaging at 4 °C for up to 9 days affected vitamins C, E, and K, folic acid, and carotenoid contents and total antioxidant capacity.

MATERIALS AND METHODS

Plant Material, Field Production, and Storage Conditions. Two spinach (*S. oleracea* L.) cultivars, 'Lazio' (flat-leafed) and 'Samish' (semisavoy or crinkle-leafed), were grown at the USDA-ARS Subtropical Agricultural Research Center farm, following commercial production practices, in Weslaco, TX (26° 08′ N, 97° 57′ W, elevation 27 m). 'Lazio'

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Table 1. Temperature and Light Conditions during Field Cultivation, Cumulative 3 Days Prior to Harvest, and Postharvest Storage of Spinach

	field cultivation[a]		cumulative 3 days prior to harvest		storage chamber	
condition	'Lazio'	'Samish'	'Lazio'	'Samish'	light	dark
mean temperature (°C)	16.3	14.8	14.9	12.3	4.04	4.04
temperature range (°C)	0.8−26.4	0.8−26.4	10.8−19.2	6.9−19.2	4−5	4−5
mean photoperiod[b] (h:min)	10:43	10:39	10:23	10:23	24	0
radiation (kW m^{-2} day^{-1})	3.4	3.2	2.9	3.3	0.14	0.00
total radiation (kW m^{-2})	245.6	207.0	8.7	9.9	*c*	0

[a] Duration between sowing and harvest for 'Lazio' and 'Samish' was 72 and 65 days, respectively. [b] Sunrise to sunset. [c] This value varies with storage duration.

and 'Samish' seeds were sown October 23 and 31, 2007, respectively, on separate beds 15 cm high, 30 cm wide at the surface, and 35 cm wide at the base. Beds were 30 m long and 1.0 m apart with three randomized beds per cultivar with an established plant density of ca. 50 plants per meter per double-row bed. Growing temperature and light conditions are described in **Table 1**. Soil texture was 47, 42, and 11% sand, silt, and clay, respectively, with 0.25% organic matter. Plants were grown under identical commercial production protocols and received uniform fertility, irrigation, and pesticide treatments. 'Lazio' and 'Samish' plants were harvested on January 3 and 4, 2008, respectively, within 2 h of sunrise from randomly selected plants from three beds (plots). Harvested plants were layered into ice chests (one ice-chest per plot) over a base layer of crushed ice covered with paper toweling. Each successive layer of plants was separated by cold, wet paper toweling. Ice chests were immediately transported (10 min) to the USDA-ARS Subtropical Agricultural Research Center laboratories in Weslaco, TX, where plants were rinsed in 4 °C reverse osmosis H_2O, dipped (30 s) in 4 °C reverse osmosis H_2O containing 0.31% sodium hypochlorite (adjusted to pH 6.8 with 1 N HCl and 0.005% Tween 20), rinsed in fresh 4 °C reverse osmosis H_2O, and then lightly patted damp-dry with paper towels. Leaves from harvested plants were immediately removed from the plant and sorted into top-canopy (leaf-blade width < 5 cm), midcanopy (leaf-blade width = 5−6.35 cm), and bottom-canopy (leaf-blade width > 6.35 cm) leaves. Each leaf-canopy group of each cultivar was placed one layer thick (15−22 g/tub) into preweighed commercial spinach retail-display clear corn-based polylactic acid resin compostable tubs (14.8 cm W × 18.7 cm L × 6 cm D) with clear snap-tight lids (EcoProducts, Boulder, CO), which were replicated three times (reps). Oxygen levels in closed tubs at 20 °C were 20.0 ± 0.03 kPa (determined by R. Saftner, USDA-ARS, Food Quality Laboratory, Beltsville, MD). Containers were placed on shelves in each of three 4 °C walk-in storage chambers, one chamber per field replicate. Tubs per shelf were either enclosed in two-layer-thick brown paper (grocery) bags (dark treatment) or exposed to retail produce-display continuous lighting provided by single-element 32 W Fluorescent Daylite Phillips model F32T8/TL841/plus alto lamps (light treatment). Lamp spectral reflectance and emittance, determined by spectral photometry (Analytical Spectral Devices Inc., Boulder, CO) adjusted to 100% reflectance using a white reference plate exposed to sunlight at solar noon, was in narrow bands within the blue (400−500 nm), green (500−600 nm), and red (600−720 nm) regions, with the two equally highest peaks occurring in the green (544 nm) and red (612 nm) regions. The average quantum flux (determined by LI-190 sensor, LI-COR, Lincoln, NE) at the top (lid) of the tub for all samples was 0.12 and 26.9 $\mu mol \cdot m^2 \cdot s$ for dark and light treatments, respectively. Chamber temperature and light conditions are presented in **Table 1**. Leaves were stored for 0, 3, 6, or 9 days and air temperatures inside the closed tubs under light and dark treatments were 4.8 and 4.6 °C, respectively. Immediately after storage, leaf fresh weight and turgidity were determined. Leaf tissue samples were frozen (liquid nitrogen) and then stored at −80 °C for < 30 days for ascorbic acid, folic acid, and phylloquinone analyses or lyophilized for carotenoid, tocopherol, and antioxidant analyses.

Leaf Turgidity and Dry Weight Measurements. Leaf turgidity at harvest was estimated by measuring leaf-blade bending using a 0° to −90° angle. Leaves, top-side up, were held at the midrib base parallel to 0° horizontal line. At harvest all leaves were turgid and parallel to the 0° horizontal line. After storage, loss of turgidity was estimated by holding the midrib base parallel to the vertex and measuring the angle of the declining midrib section. Leaf dry weight was determined as a percentage of fresh tissue after lyophilization.

Compositional Analyses. *Ascorbic Acid.* Ascorbic acid and dehydroascorbic acid were extracted from 3 g of frozen leaf tissue and determined spectrophotometrically at 525 nm according to the procedure of Hodges et al. (*16*).

Carotenoids and Tocopherols. Samples (0.05 g of ground, freeze-dried tissue) were weighed into a 15 mL glass tube, and 7.5 mL of 1% butylated hydroxytoluene (BHT) in ethanol, and 500 μL of the internal standard (120 μM *trans*-β-apo-8 carotenal) was added. The tube was capped under a stream of N_2 and sonicated. Samples were then placed in a 70 °C dry bath for 15 min, after which 180 μL of 80% KOH was added. Tubes were again capped under a stream of N_2 and sonicated to mix, followed by placing them in a 70 °C dry bath for 30 min. Tubes were then removed and cooled for 5−10 min at room temperature and 3.0 mL of Milli-Q water (Millipore, Bedford, MA) and 3.0 mL of hexane/toluene solution (10:8 v/v) added. Tubes were vortexed and centrifuged at 4 °C for 5 min at 4000g_n. The organic layer was removed to a clean 8 mL glass culture tube, which was immediately placed under a stream of N_2 in a water bath set at 30 °C. Extractions with the hexane/toluene mix (10:8) were repeated four times, and the organic fraction was completely dried. The dried pellet was then dissolved in 500 μL of 100% acetone. Samples were then filtered into HPLC vials with 0.2 μm nylon filters (Millipore Corp., Bedford, MA) and a glass syringe. The constituent carotenoids and tocopherols were separated on a photodiode array HPLC (Waters Corp., Milford, MA) on a C18 column (Luna 5 μm 150 × 4.6 mm i.d., Phenonomex, Torrance, CA) using acetonitrile/ethanol (50:50 v/v) at a flow rate of 1.2 mL/min for 20 min. Absorbance was measured at 450 and 290 nm with a scan between 200 and 500 nm. The carotenoids were quantified at 450 nm and tocopherols at 290 nm using previously developed standard curves for each compound.

Folate. Folate, as 5-methyltetrahydrofolate, was extracted from 1 g of frozen leaf tissue and measured with fluorescence HPLC at 290 nm excitation, 350 nm emission, and a photomultiplier gain setting of 12 according to the procedure of Lester et al. (*17*).

Phylloquinone. Phylloquinone (vitamin K_1) was extracted under low light at 4 °C according to the modified procedure of Booth et.al. (*18*). One gram of frozen leaf tissue was homogenized (Kinematica GmBH polytron, Sweden) at medium speed in 10 mL of H_2O containing 200 μg/mL menaquinone (K_2) internal standard for 1 min. Fifteen milliliters of 2-propanol/hexane (3:2 v/v) was added, homogenized 1 min, then centrifuged 5 min at 1500g_n. The top hexane layer was removed and evaporated to dryness under a N_2 stream and then redissolved in 4 mL of hexane. Preconditioned (4 mL of 3.5% ethyl ether in hexane, followed by 4 mL of 100% hexane) silica gel columns (BakerBond spe columns; Mallinckrodt-Baker, Phillipsburg, NJ) were loaded with 1 mL of extract and washed with 2 mL of hexane. Phylloquinone was eluted with 8 mL of 3.5% ethyl ether in hexane, and the eluate was evaporated to dryness with low heat (40 °C) under N_2 and then redissolved in 2 mL of HPLC mobile phase (99% methanol w/1% 0.05 M sodium acetate, pH 3.0, buffer; pH was adjusted with acetic acid) and filtered through a 0.2 μm nylon filter (Millipore Corp.). Separation using HPLC was accomplished on a Vydac 201 TPh54 5 μm column (250 mm × 4.6 mm i.d.) from W. R. Grace Vydac Co. (Columbia, MD) with a flow rate of 1 mL/min. Detection of phylloquinone absorbance was at 270 nm UV. All chemicals were obtained through Sigma Chemical Co. (St. Louis, MO).

Oxygen Radical Absorbing Capacity (*ORAC*). The total antioxidant capacity (ORAC) in 50−100 mg of freeze-dried tissue was analyzed on a Fluoroskan Ascent FL microplate reader (Thermo Electron Corp., Vantaa, Finland) using 2,2′-azobis(2-amidinopropane) dihydrochloride

(AAPH) as a peroxyl generator and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as a standard according to the method of Prior et al. (*19*).

Statistical Analyses. Data from leaf turgidity estimates, dry weight, compositional analyses, and ORAC determinants were then subjected to analysis of variance using the general linear model of SAS (SAS Institute Inc., Cary, NC). Means were compared using the "GLIMMIX" procedure of SAS version 9.1. Only $P \leq 0.05$ significant differences are discussed unless stated otherwise. The experiment-wise analysis is based upon the analysis of variance (ANOVA) of the factorial design (cultivar × leaf position × days in storage × light level) by the general linear model of SAS version 9.1.

RESULTS

Leaf Characterization at Harvest. Seventy-two-day-old 'Lazio' plants had 21.8 ± 0.5 leaves and a total plant leaf area of 1594 ± 63 cm^2. Sixty-five-day-old 'Samish' plants had 20.7 ± 0.8 leaves and a total plant leaf area of 1572 ± 49 cm^2. Leaves with an area of < 3 cm^2 were not counted. 'Lazio' and 'Samish' leaves were not different in average leaf fresh and dry weights, leaf area, or leaf area expressed on a dry weight basis (data not shown). As 'Lazio' leaves became older, progressing from top-canopy to bottom-canopy leaves, leaf area increased linearly. 'Lazio' leaf fresh weight increased, whereas average percent leaf dry weight decreased with leaf age. Middle-aged (midcanopy) 'Samish' leaves had the highest average percent leaf dry weight, and average leaf area compared to younger (top-canopy) and older (bottom-canopy) leaves.

Leaf Dry Weight and Turgidity after Storage. Leaf percent dry weight at harvest was 20−23% higher in 'Lazio' than in 'Samish' and remained relatively unchanged for both cultivars, as did leaf turgidity following 9 days in continuous darkness (**Figure 1**). Total percent change in dry weight of leaves exposed to 3 days of continuous light also remained relatively unchanged, indicating little fresh weight loss occurred during this period. By day 9 under continuous light, dry weights increased (i.e., fresh weight loss) an average of 18% ('Lazio') and 24% ('Samish'), with the greatest increases occurring in baby-leaf (top- and midcanopy) versus larger (bottom-canopy) leaves. Although 'Samish' had the greatest percent increase in dry weight (i.e., fresh weight loss) under continuous light, this crinkle-leaf structure experienced relatively little loss in leaf turgidity. Smooth-leaf 'Lazio', however, had a noticeable loss of leaf turgidity by 3 days under continuous light, with top-canopy leaves showing a linear decline in turgidity throughout storage, and mid- and bottom-canopy leaves showing no additional loss of turgidity by 6 days, although values declined again following 9 days of storage.

Total and Free Ascorbic Acid. Total and free ascorbic acid concentrations were similar in 'Lazio' and 'Samish', with top-canopy leaves having higher concentrations than midcanopy leaves, which were higher than bottom-canopy leaves (**Figure 1**). All leaves exposed to continuous light had higher concentrations of total and free ascorbic acid than those exposed to darkness during the first 6 days of storage. Only total ascorbic acid concentrations in 'Samish' leaves exposed to light were higher after 9 days than those in the dark leaves. Total ascorbic acid concentration in all leaves, light or dark, after 9 days of storage returned to initial storage levels. However, free ascorbic acid, particularly in top-canopy leaves, was much lower after 9 days storage, indicating top-canopy leaves were under greater stress as reflected by their higher dehydroascorbic acid levels (data not shown), which is the difference between total and free ascorbic acid levels.

Folate. Folate content increased by 84 to 100% in 'Lazio' and 'Samish' leaves, respectively, under continuous light by 9 days of storage (**Figure 2**). However, 9 days of continuous darkness resulted in folate content either decreasing ('Lazio') or remaining mostly unchanged ('Samish'). Light-exposed leaves of 'Lazio' versus 'Samish' were higher in folate, with top-canopy leaves having higher levels than midcanopy, which had higher levels than bottom-canopy leaves. 'Samish' top- and midcanopy leaves were similar in folate content, with both being similar to bottom-canopy leaves until day 9, when folate content in bottom-canopy leaves remained unchanged, whereas baby-leaf sizes experienced a further increase.

Phylloquinone. Phylloquinone content increased continuously under illuminated storage by as much as 50−100% in 'Samish' and 'Lazio' leaves (**Figure 2**). Phylloquinone also increased in leaves under continuous darkness, but by no more than 35%. However, under continuous darkness phylloquinone content first increased within the first 6 days and thereafter declined. Top-canopy leaves had the highest levels of phylloquinone followed by midcanopy leaves, and the lowest levels were generally found in bottom-canopy leaves. Only top-canopy leaves had consistently higher levels than their counterpart leaves stored in darkness.

Tocopherols. α- and γ-tocopherols in 'Lazio' and 'Samish' were generally higher in light-stored leaves than in the dark-stored leaves (**Figure 2**). During storage, little change occurred in either α- or γ-tocopherol concentrations until 9 days, when both tocopherol types increased in light-stored top- and midcanopy leaves. Bottom-canopy leaves saw either little change or a dramatic concentration change, especially in 'Samish', over storage time. One notable finding was the inverse levels of α- compared to γ-tocopherol in bottom-canopy leaves compared to top- and midcanopy leaves. α-Tocopherol content in both cultivars was higher in bottom-canopy (older) leaves than in younger mid- and top-canopy leaves. The exact reverse of this leaf canopy position with respect to concentration was found with γ-tocopherol.

Carotenoids. Lutein/zeaxanthin content was higher in leaves stored under light versus darkness (**Figure 3**). 'Lazio' had higher lutein/zeaxanthin concentrations in top- and midcanopy than bottom-canopy leaves, whereas 'Samish' had higher concentrations in mid- and bottom-canopy than top-canopy leaves. β-Carotene and violaxanthin concentrations were lowest in top- versus mid- and bottom-canopy leaves and were lowest in light- versus dark-exposed leaves (**Figure 3**).

Oxygen Radical Absorbing Capacity (Total Antioxidants). Antioxidant capacity increased during storage, with no consistent difference between leaves stored in continuous light versus darkness, except for top-canopy leaves (**Figure 3**). Top-canopy leaves of 'Lazio' under continuous light had higher antioxidant capacity starting from storage day 6 onward than dark-stored leaves, whereas 'Samish' leaves under continuous light had higher levels from day 3 onward. Total antioxidant capacity was higher in 'Samish' versus 'Lazio' top-canopy leaves.

Analysis of Variance (ANOVA). Cultivar responses were significant for all vitamins and carotenoids except α-tocopherols. The full model was therefore reduced and analyzed by cultivar with the simplified ANOVA for 'Lazio' (**Table 2**) and 'Samish' (**Table 3**) with polynomial fits for leaf position (node 6 for bottom-, node 12 for mid-, and node 18 for top-canopy leaves) and for time (days) held in storage.

DISCUSSION

Turgidity and Percent Dry Weight. Storage of top-canopy (young), midcanopy (medium-aged), and bottom-canopy (oldest) spinach leaves under continuous darkness at 4 °C resulted in no decline in turgidity over the 9 day storage period (**Figure 3**). This result corroborates a previous study of "young" versus "old" spinach leaves, stored under dark conditions, that found no loss in leaf turgidity following 9 days at 2 °C (*20*). In contrast, a

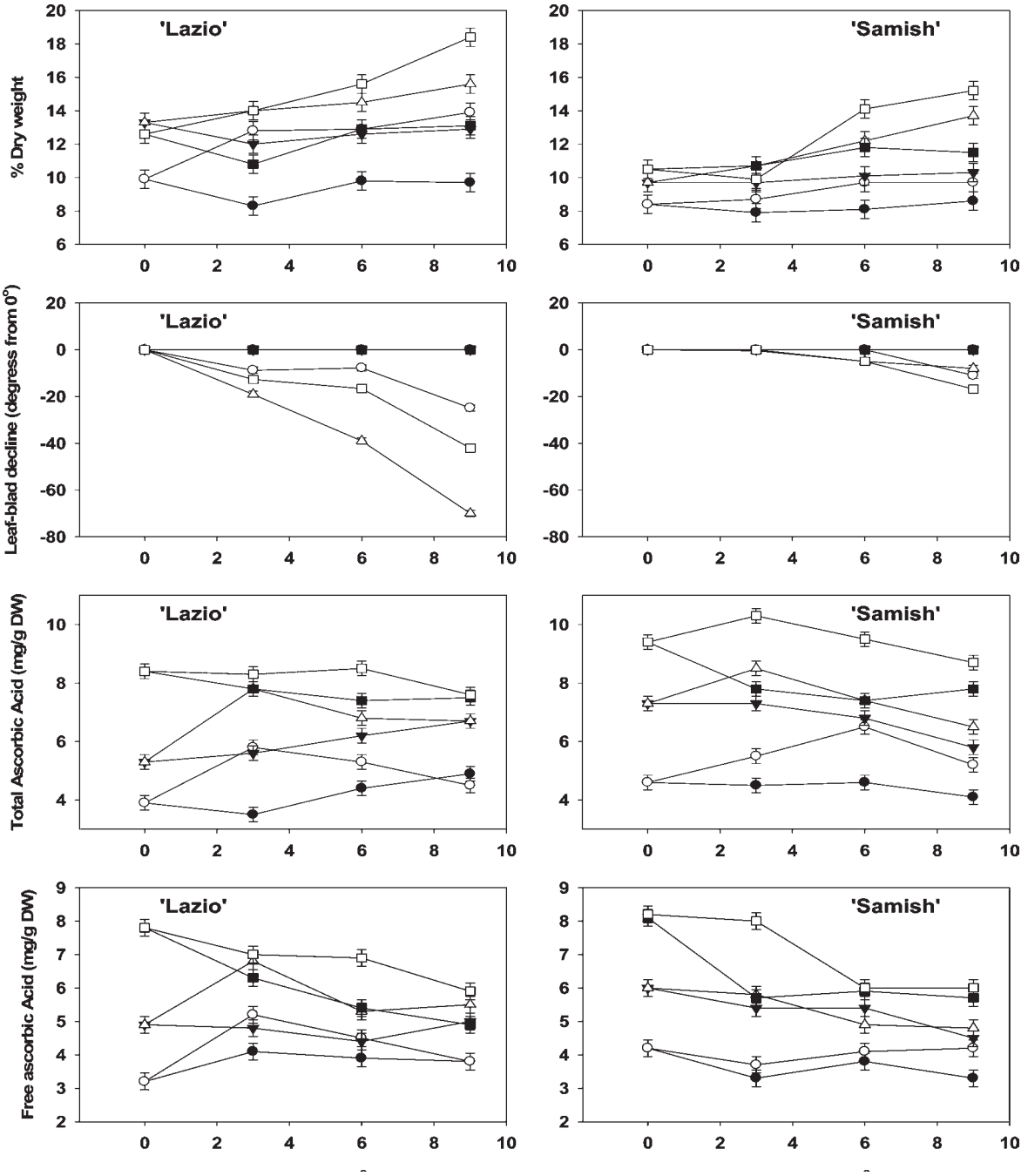

Figure 1. Top-canopy leaves held in continuous light (□) or darkness (■), midcanopy leaves held in continuous light (△) or darkness (▲), and bottom-canopy leaves held in continuous light (○) or darkness (●). Values are means ± standard deviation of three field plots each determined as a mean of three replications.

substantial decline in turgidity by 9 days of storage occurred when leaves were placed under continuous light. Top-canopy leaves had a greater decline in turgidity compared to midcanopy, which had a greater decline ('Lazio') than, or equal to ('Samish'), bottom-canopy leaves. The degree of leaf turgidity decline (wilt) under continuous light and the lack of wilting under continuous darkness paralleled increased leaf percent dry weight or fresh weight loss (**Figure 1**). Leaf moisture loss within a closed package system under continuous light may be explained by photolysis of H_2O and fixing of CO_2 (photosynthesis), which can also result in increased photosynthetic associated levels of some carotenoids and the vitamins C, K, E, and B_9 (*21*−*25*) provided sufficient light intensity. However, the exact physiological mechanism remains to be determined.

Vitamin C (Ascorbic Acid). Increasing photon flux density (light intensity) has been noted to increase total vitamin C in spinach during field production (*26*). Previous postharvest storage studies of spinach leaves held in continuous darkness found either decreasing (*16*) or static levels, with static levels of total vitamin C being dependent upon both temperature (retention at 2 °C while decreasing at 10 °C) and storage duration (retention at 6 days or less, but decreasing beyond 6 days) (*27*). Our study found that total vitamin C concentrations in spinach leaves exposed to continuous light for 9 days was retained (**Figure 1**). More specifically, an increase in total vitamin C occurred in leaves stored in light by 3 days before retreating to 0 day levels by day 9. Our findings are corroborated by a similar increase in total vitamin C in light-stored barley (*Hordeum vulgar*) leaves (*28*).

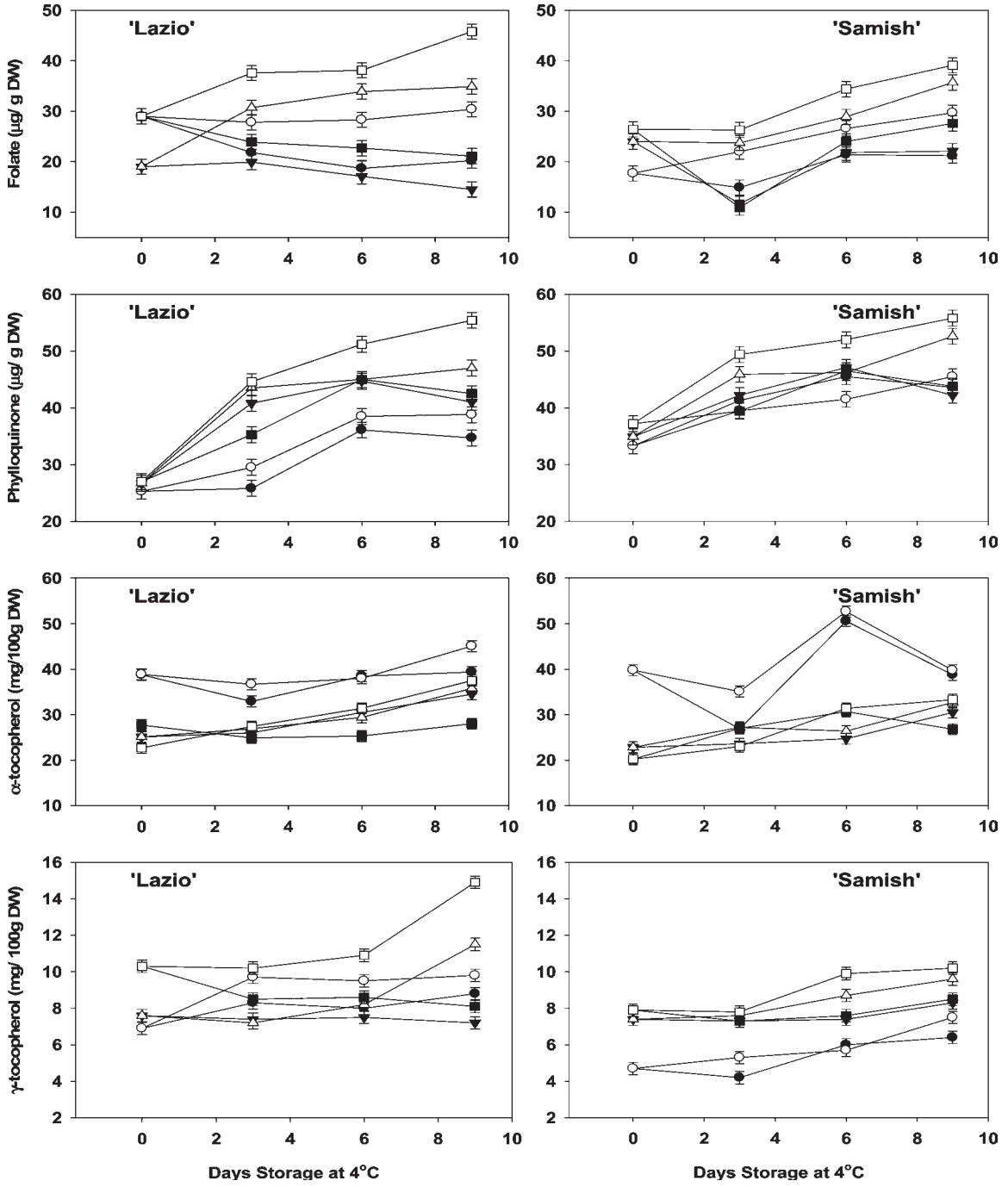

Figure 2. Top-canopy leaves held in continuous light (□) or darkness (■), midcanopy leaves held in continuous light (△) or darkness (▲), and bottom-canopy leaves held in continuous light (○) or darkness (●). Values are means ± standard deviation of three field plots each determined as a mean of three replications.

Free ascorbic acid levels in our study were also retained in light-stored leaves, particularly from mid- and bottom-canopy leaves (**Figure 1**). Only top-canopy leaves had lower levels of free ascorbic acid at 9 days compared to 0 days of storage, with 'Samish' leaves retaining free ascorbic acid longer, until 6 days, then declining, whereas 'Lazio' declined by 3 days and again by 9 days. Although top-canopy leaves demonstrated a decline in free ascorbic acid during storage, they always maintained a higher concentration than mid- or bottom-canopy leaves. Bergquist et al. (*20*) had similar findings when comparing young (18 days old) versus old (30 days old) leaves. Toledo et al. (*29*) also reported more free ascorbic acid in light- versus dark-stored spinach leaves. It was expected that in our study top-canopy (younger) leaves would have higher ascorbic acid content than older mid- and bottom-canopy leaves, as high levels of vitamin C are associated with young, rapidly growing regions in plant canopies (*26*).

Vitamin B_9 (Folate). A previous postharvest storage study of spinach leaves held in the dark for 7 days at 4 °C reported a ~30% folate decline (*30*). Our study, using 'Lazio' leaves, showed a similar decline in folate content in dark-stored leaves after 9 days, but little or no decline occurred in similarly stored 'Samish' leaves (**Figure 2**). Our study additionally showed that this loss in folate could be prevented if leaves were stored in continuous light. Additionally, top-canopy (younger) had higher folate concentrations than bottom-canopy (older) leaves, which is consistent with

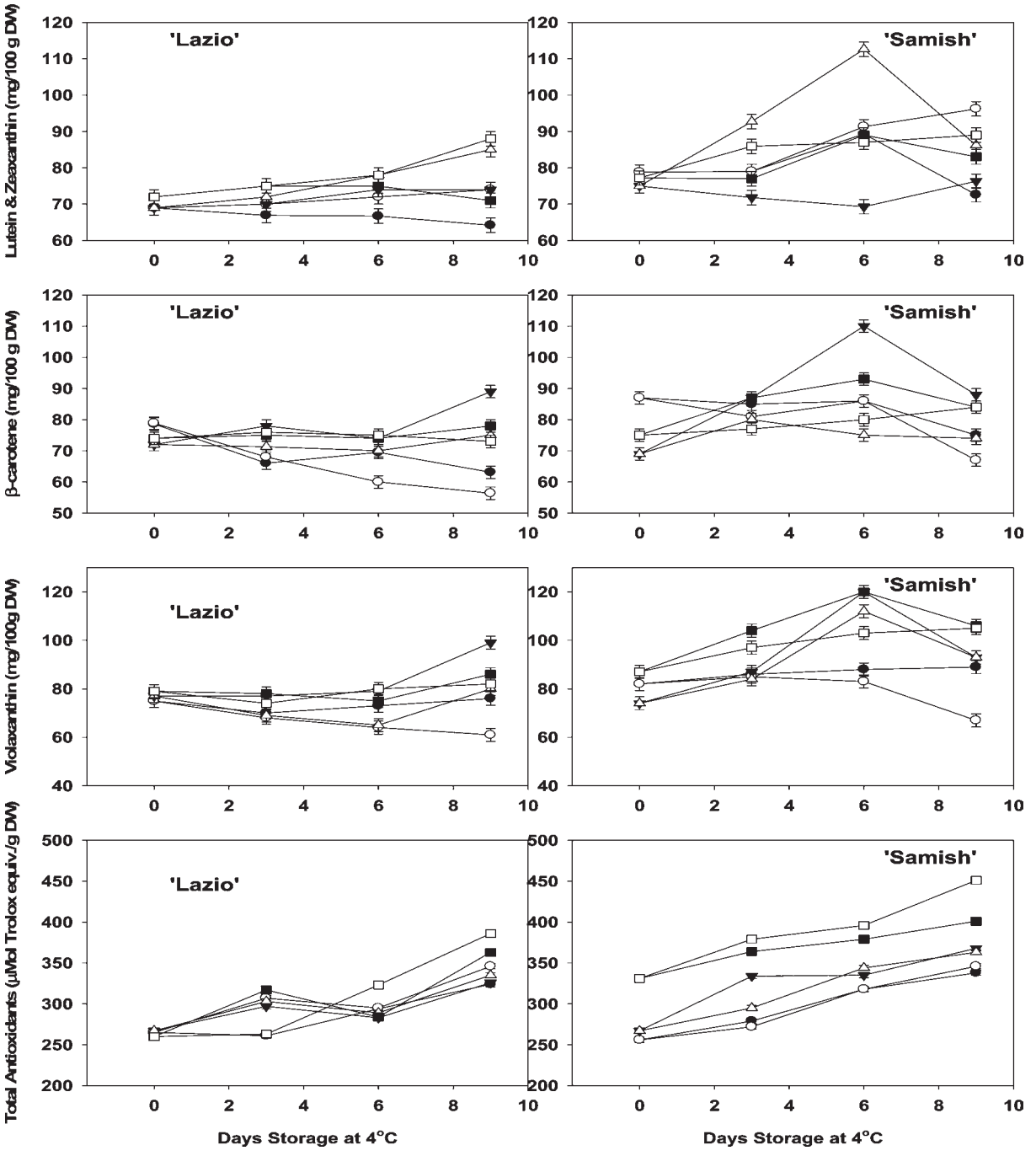

Figure 3. Top-canopy leaves held in continuous light (□) or darkness (■), midcanopy leaves held in continuous light (△) or darkness (▲), and bottom-canopy leaves held in continuous light (○) or darkness (●). Values are means ± standard deviation of three field plots each determined as a mean of three replications.

previous studies (*31*). Folate in the current study fluctuated depending on the nature of the tissue as evidenced by cultivar, leaf age (younger > older), and storage light exposure (light > dark).

Vitamin K_1 (Phylloquinone). It has been shown that growing location, climate, soil condition, stage of maturation, cultivar, and processing can all influence the phylloquinone content of green leafy vegetables (*32*, *33*). However, little is known about the postharvest fate of phylloquinone in fresh produce. The current study showed that phylloquinone increased with days of storage in both dark- and light-stored spinach leaves and that light-stored leaves generally had higher levels of phylloquinone than dark-stored leaves (**Figure 2**). The exact physiology behind increased levels of phylloquinone remains to be determined.

Provitamin A (*β*-Carotene) and Xanthopylls. Unlike other chloroplast-bound human bioactive compounds, carotenogenesis, although stimulated by light, it is not always required for its induction (*34*). Our findings with spinach leaves demonstrated little difference in the concentrations of xanthophylls (lutein, zeaxanthin, and violaxanthin) and *β*-carotene when stored under light versus dark conditions, which corroborates that either carotenogenesis is light-independent or the threshold whereby carotenogensis is stimulated has not been crossed (**Figure 3**). However, reversible changes in xanthophylls under light and dark conditions occur (*35*). This light-dependent

Table 2. 'Lazio' Analysis of Variance for Dry Weight (DW), Ascorbic Acid (Ascorbate), Carotenoids, Folate, Phylloquinone (Vitamin K_1), α- and γ-Tocopherol, Oxygen Radical Absorbing Capacity (ORAC), and Turgidity[a]

		ascorbate			carotenoids					tocopherol			
source	DW (%)	total	free	dehydro	violaxanthin	lutein and zeaxanthin	β- carotene	folate	vitamin K_1	α	γ	ORAC	turgidity
leaf (Lf)	**	**	**	**	**	*	NS	NS	NS	**	*	NS	**
days (D)	**	**	NS	**	NS	NS	NS	*	**	**	**	**	**
light (L)	**	**	**	**	NS	*	NS	**	NS	NS	**	NS	**
Lf × D	NS	NS	NS	**	NS	NS	*	*	NS	NS	NS	NS	*
Lf × L	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	*	NS	**
D × L	NS	*	**	NS	NS	NS	NS	NS	*	NS	NS	NS	**
Lf × D × L	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
rep	NS	*	*	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
Lf	L**	L**	L**	L**	L**	L*	L*	–	–	L*	L**	–	Q**
days	L**	L**	–	Q**	Q*	–	–	Q**	Q**	L**	–	L**	L**

[a] Relationships between leaf canopy position (leaf) and days in storage (days) under ambient light of dark conditions (light) are shown as polynomial fits, where significant. NS, *, ** = not significant and significant at $P = 0.05$ or $P = 0.01$, respectively. L, Q, and – represent linear, quadratic, or nonsignificant polynomial relationships, respectively.

Table 3. 'Samish' Analysis of Variance for Dry Weight (DW), Ascorbic Acid (Ascorbate), Carotenoids, Folate, Phylloquinone (Vitamin K_1), α- and γ-Tocopherol, Oxygen Radical Absorbing Capacity (ORAC), and Turgidity[a]

		ascorbate			carotenoids					tocopherol			
source	DW (%)	total	free	dehydro	violaxanthin	lutein and zeaxanthin	β- carotene	folate	vitamin K_1	α	γ	ORAC	turgidity
leaf (Lf)	**	**	**	**	**	NS	NS	NS	**	**	NS	**	NS
days (D)	**	*	*	**	NS	NS	NS	**	NS	NS	**	**	*
light (L)	**	**	**	**	NS	*	NS	**	NS	NS	**	NS	NS
Lf × D	**	NS	NS	*	NS	NS	NS	NS	NS	NS	*	NS	NS
Lf × L	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS
D × L	**	NS	NS	**	*	*	*	NS	**	NS	**	NS	NS
Lf × D × L	**	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS
rep	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Lf	L**	L**	L**	L**	L**	L*	–	L**	L*	L**	–	L**	–
days	L**	L**	L*	L** C*	–	C*	–	L** C*	–	L*	L**	L**	L**

[a] Relationships between leaf canopy position (leaf) and days in storage (days) under ambient light of dark conditions (light) are shown as polynomial fits, where significant. NS, *, ** = not significant and significant at $P = 0.05$ or $P = 0.01$, respectively. L, C, and – represent linear, cubic, or nonsignificant polynomial relationships, respectively.

conversion of xanthophylls affects the violaxanthin cycle, whereby under lighted conditions violaxanthin is converted to zeaxanthin and under darkened conditions zeaxanthin is converted to violaxanthin. This light-dependent violaxanthin cycle likely occurred in our study, reducing the zeaxanthin levels in light-stored leaves while increasing the violaxanthin levels in dark-stored leaves, thus resulting in the minimized levels of xanthophylls in light- versus dark-stored spinach leaves.

Vitamin E (α-Tocopherol) and γ-Tocopherol. γ-Tocopherol is the biosynthetic pathway precursor to α-tocopherol, the predominant tocopherol in plant tissues and the molecular form containing nearly all of the vitamin E activity (*36*). α-Tocopherol concentrations in plant tissues are inversely related to growth rate, with the highest concentrations found in "slower-growing" older leaves versus "faster-growing" younger leaves (*37*). Our spinach leaf study corroborated this as α-tocopherol was found to be higher in bottom-canopy (older) leaves than in mid- or top-canopy (younger) leaves (**Figure 2**). Coincidentally, γ-tocopherol was found to be higher in young leaves than in older leaves (**Figure 2**), possibly due to γ-tocopherol methyltransferase activity being more susceptible to oxidative stress in younger versus older leaves, thus affecting the biosynthetic conversion of γ-tocopherol to α-tocopherol (*37*).

Oxygen Radical Absorbing Capacity (Total Antioxidants). It is known that younger (baby-leaf sized) spinach leaves have higher levels of total antioxidants than older leaves (*38*). In our study the younger top- and midcanopy leaves (both baby-leafed sized) versus the older bottom-canopy leaves were found to have higher levels of total antioxidants, especially for the cultivar 'Samish' (**Figure 3**). 'Lazio' overall had lower levels of antioxidants than 'Samish', which may be due to the relatively lower levels of carotenoids and tocopherols in 'Lazio' versus 'Samish'. Differences in phenolic contents may also be present. However, 'Lazio' tended to have higher levels of total antioxidants in mid- and top-canopy leaves with days in storage, which likely is coincident with higher levels of the powerful antioxidant carotenoids and tocopherols following 6 days of storage.

In conclusion, although simulated retail light conditions in this study were found to be beneficial in maintaining/enhancing essential human-health vitamins C, B_9, K_1, and E and the carotenoids lutein, violaxanthin, zeaxanthin, and β-carotene over time, storage in light contributed to some leaf wilting after 3 days of storage ('Lazio' > 'Samish' and baby-leafed size > older leaves) as measured by leaf blade bending. Leaf maturity, regardless of cultivar, was a major determinant in the aforementioned essential human-health compound concentrations, with younger baby-leafed sized leaves generally having higher levels of vitamins C, B_9, and K_1 and the carotenoids lutein, violaxanthin, zeaxanthin, and β-carotene. The differences in human-health bioactive compounds measured in this study would not be visually apparent to the consumer, whereas some wilting may impact consumer acceptance. Focusing on continuous light exposure during retail display combined with specific cultivars (e.g., crinkled-leafed types) and leaf maturity (i.e., baby-leafed size) appears to be the strategy to preserving and enhancing the concentration of spinach-derived human-health bioactive compounds.

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