JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 4901–4906 4901
DOI:10.1021/jf100146m

γ-Irradiation Dose: Effects on Baby-Leaf Spinach Ascorbic Acid, Carotenoids, Folate, α-Tocopherol, and Phylloquinone Concentrations

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Ionizing radiation of fruits and vegetables, in the form of γ rays or electron beams, is effective in overcoming quarantine barriers in trade and prolonging shelf life, but a void of information persists on ionizing radiation effects of vitamin profiles in individual foods. Baby-leaf spinach from commercial cultivars, flat-leafed 'Lazio' and crinkled-leaf 'Samish', was grown, harvested, and surface sanitized according to industry practices. Baby-leaf spinach of each cultivar was packaged under air or N_2 atmosphere, representing industry practices, then exposed to cesium-137 γ-radiation at 0.0, 0.5, 1.0, 1.5, or 2.0 kGy. Following irradiation, leaf tissues were assayed for vitamin (C, E, K, B_9) and carotenoid (lutein/zeaxanthin, neoxanthin, violoxanthin, and β-carotene) concentrations. Atmospheres by irradiation had little consistent effect, but N_2 versus air was associated with elevated dihydroascorbic acid levels. Four phytonutrients (vitamins B_9, E, and K and neoxanthin) exhibited little or no change in concentration with increasing doses of irradiation. However, total ascorbic acid (vitamin C), free ascorbic acid, lutein/zeaxanthin, violaxanthin, and β-carotene all were significantly reduced at 2.0 kGy and, depending on cultivar, were affected at lesser doses of 0.5 and 1.5 kGy. Dihydroascorbic acid, the most affected compound and an indicator of stress, likely due to irradiation-generated oxidative radicals, increased with increasing irradiation doses >0.5 kGy.

KEYWORDS: Spinach (*Spinacia oleracea*); neoxanthin; violaxanthin; lutein; zeaxanthin; β-carotene; 5-methyltetrahydrofolate; growing/sampling

INTRODUCTION

In the wake of numerous outbreaks of foodborne illness in recent years involving spinach, the U.S. Food and Drug Administration (FDA) announced in August 2008 that it will allow the irradiation of fresh iceberg lettuce and spinach to kill *Escherichia coli* O157:H7 and *Salmonella enteric* (Federal Register Final Rule - 73 FR49583 August 22, 2008). Pathogenic bacteria can easily become internalized in leaf tissues of iceberg lettuce and spinach and are protected from the antimicrobial effects of surface treatments. A property of γ radiation is its high penetration, which allows food tissue decontamination (*1*) and the processing of foods in the final packages, minimizing the possibility of cross-contamination. The FDA allows irradiation of retail iceberg lettuce and spinach with a dose of up to 4 kGy in response to a petition filed by The National Food Processors Association on behalf of The Food Irradiation Coalition. Although the FDA considers food irradiation to be a safe technology, concerns include the potential to form bioactive compounds and the possibility to deplete phytonutrients (*2*). Irradiation has been extensively studied as a food-processing technology; however, the use of the process against internalized bacteria has only recently emerged (*1*, *3*−*6*). The FDA in its ruling recognizes that spinach is an excellent source of human nutrients, vitamins A (as carotenes), C, K, and B_9 (folate), and that a dose of up to 4 kGy "will not have an adverse impact on the nutritional adequacy" of spinach. However, FDA regulation is often based on leafy greens other than spinach or literature more than 40 years old. Recent studies show that spinach requires a dose of >1.06 kGy to achieve undetectable levels of *E. coli* O15:H7 (*3*), but a dose of 1.0 kGy immediately decreases ascorbic acid levels (vitamin C) by as much as 25% (*4*). Although much of the aforementioned emerging literature addresses the efficacy on enteric bacteria of spinach, it does little to address the potential to deplete phytonutrients. Also, few, if any, irradiation studies use freshly harvested plant material (e.g., spinach) of known cultivars or document the crop production details or the atmospheres surrounding the produce prior to irradiation treatments.

The purpose of this study was to determine the effect of increasing doses of irradiation (0, 0.5, 1.0, 1.5, and 2.0 kGy) on

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Published on Web 03/24/2010

vitamin C, E, K, and B_9 and carotenoid contents of baby-leaf spinach of flat-leaf 'Lazio' and crinkled-leaf 'Samish' types enclosed in retail packaging (N_2) or air atmospheres.

MATERIALS AND METHODS

Plant Material and Field Production. Two spinach (*Spinacia oleracea* L.) cultivars, 'Lazio' (flat-leafed) and 'Samish' (semisavoy or crinkled-leafed), were grown at the USDA-ARS Subtropical Agricultural Research Center, using commercial production practices, in Weslaco, TX (26° 08′ N, 97° 57′ W, elevation 27 m). 'Lazio' and 'Samish' seeds were sown November 23 and 30, 2008, 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 four randomized beds per cultivar with an established plant density of ca. 50 plants per meter per double-row bed. Soil texture was 20% sand, 34% silt, and 46% clay, with 0.63% 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 26−29, 2009, within 2 h of sunrise from randomly selected plants from four beds (i.e., four replicates per cultivar). Harvested plants were layered into ice chests (one ice-chest per replicate) over a base layer of crushed ice covered with cold, wet paper toweling. Each successive layer of plants was separated by cold, wet paper toweling. Ice chests were immediately transported (10 min) to the laboratory in Weslaco, TX, where plants were rinsed in 4 °C reverse osmosis water, 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 baby-leaf (leaf-blade width ≤ 5 cm) leaves. Twenty-five grams of leaf tissue was placed in 1 L clear, sealable polyethylene bags with one bag per cultivar ($n = 2$), per irradiation treatment ($n = 5$), per atmosphere treatment ($n = 2$), per replicate ($n = 4$); total $n = 80$.

Atmospheres. Nitrogen atmosphere treatments were achieved by flushing bags with ultrahigh-purity nitrogen and then sealed. Air treatments were bags sealed without being flushed with nitrogen. Oxygen (O_2) and carbon dioxide (CO_2) measurements within all bags were determined by a zirconia sensor for percent O_2 and an infrared sensor for percent CO_2 by a headspace gas analyzer (Dansensor Checkmate II, Glen Rock, NJ). Prior to irradiation, mean O_2 and CO_2 levels in air bags were 20.4 and 0.45% and in N_2 flushed bags, 1.5 and 0.05%, respectively. Postirradiation mean O_2 and CO_2 levels in air bags were 16.0 and 3.5% and in N_2 flushed bags, 0.5 and 2.2%, respectively.

Irradiation. Bags were placed in irradiation cylinders and kept on ice in the dark for the 50 min transportation to the USDA-APHIS irradiation facility Moore Air Field, Mission, TX. During irradiation all cylinders were removed from the ice chest and placed in double-layered brown paper bags pre- and postirradiation. Irradiation was performed using a ^{137}Cs source (Husman model 521A, Isomedix, Inc., Whippany, NJ), dose rate 2.4 kGy/h at doses of 0.0, 0.5, 1.0, 1.5, and 2.0 kGy at 21 °C. All bags including the nonirradiated control were held at 21 °C for 60 min including irradiation time to equalize time at 21 °C.

Routine dosimetry was conducted with radiochromic dosimeters (FWT-70, Opti-Chromic, Far West Technology, Goleta, CA) placed one per bag on the outside of the spinach bags and read at a wavelength of 656 nm (model FWT-200S, Far West Technology). Transfer reference standard dosimetry was done with the Fricke system (Nordion, Kanata, ON, Canada).

Irradiated and nonirradiated spinach was placed on ice in the dark and transported back to Weslaco, TX, where samples were either frozen (liquid nitrogen) and then stored at −80 °C for < 30 days for folate and phylloquinone analyses or lyophilized for percent dry weight, carotenoid, and tocopherol analyses. Ascorbic acid analysis was done on fresh, nonfrozen tissue.

Compositional Analyses. All chemicals and standards unless otherwise stated were obtained through Sigma Chemical Co. (St. Louis, MO).

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. (*7*).

Carotenoids and Tocopherols. Samples (0.05 g of 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 (86.82 μM *trans*-β-apo-8 carotenal) added. Tissues were homogenized for 15 s at speed 4 using a homogenizer fitted with a PT 10/35 probe (Brinkman Instruments Inc. Westbury, NY) and then transferred to 15 mL screw-cap vial. The vial 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. Vials 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. Vials 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. Vials were vortexed and left at room temperature for phase separation (10 min). 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.) with a glass syringe. The constituent carotenoids and tocopherols were separated on a photodiode array HPLC (Waters Corp., Milford, MA) on a C18 column (Discovery 5 μm, 150 × 4.6 mm i.d., Sulpelco, Bellefonte, PA) using acetonitrile/ethanol (50:50 v/v) at a flow rate of 1.0 mL/min for 10 min. Absorbance was measured at 290 and 454 nm with a scan between 200 and 500 nm. The tocopherols at 290 nm and carotenoids at 454 nm were quantified using previously developed standard curves for each compound. Carotenoid separation and retention times are portrayed in **Figure 1**. Standards and standard curves were supplied by D. M. Hodges, AgriFood Canada, Nova Scotia, Canada.

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. (*8*).

Phylloquinone. Phylloquinone (vitamin K_1) was extracted under low light at 4 °C according to the modified procedure of Booth et al. (*9*). 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 for 1 min, and then centrifuged for 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; the 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.

Statistical Analyses. Analysis of variance of the factorial design was done with the general linear model (SAS, ver. 9.1) and used for the regression polynomial fits. Mean comparisons were made using LSD and the differences reported at $P \leq 0.01$. Unless stated otherwise, only results significant at $\alpha \leq 0.01$ are discussed.

RESULTS AND DISCUSSION

Dosimetry. Mean absorbed doses were 4−8% higher than the target dose and the dose uniformity ratio (maximum absorbed dose/minimum absorbed dose) was ∼1.3 for all doses (**Table 1**).

Gas Atmospheres. Responses of 'Lazio' and 'Samish' baby-leaf spinach, carotenoids, vitamins B_9, E, and K, on a dry weight basis, and ascorbic acid on a fresh weight basis to irradiation dose and bag atmosphere treatments demonstrated that carotenoids and vitamins B_9, E, and K were not affected by irradiation up to 2.0 kGy (**Table 2**). Also, treatment bag atmosphere had essentially no impact, except for ascorbic acid. Nor was there a significant interaction with bag atmosphere and irradiation on β-carotene, neoxanthin,

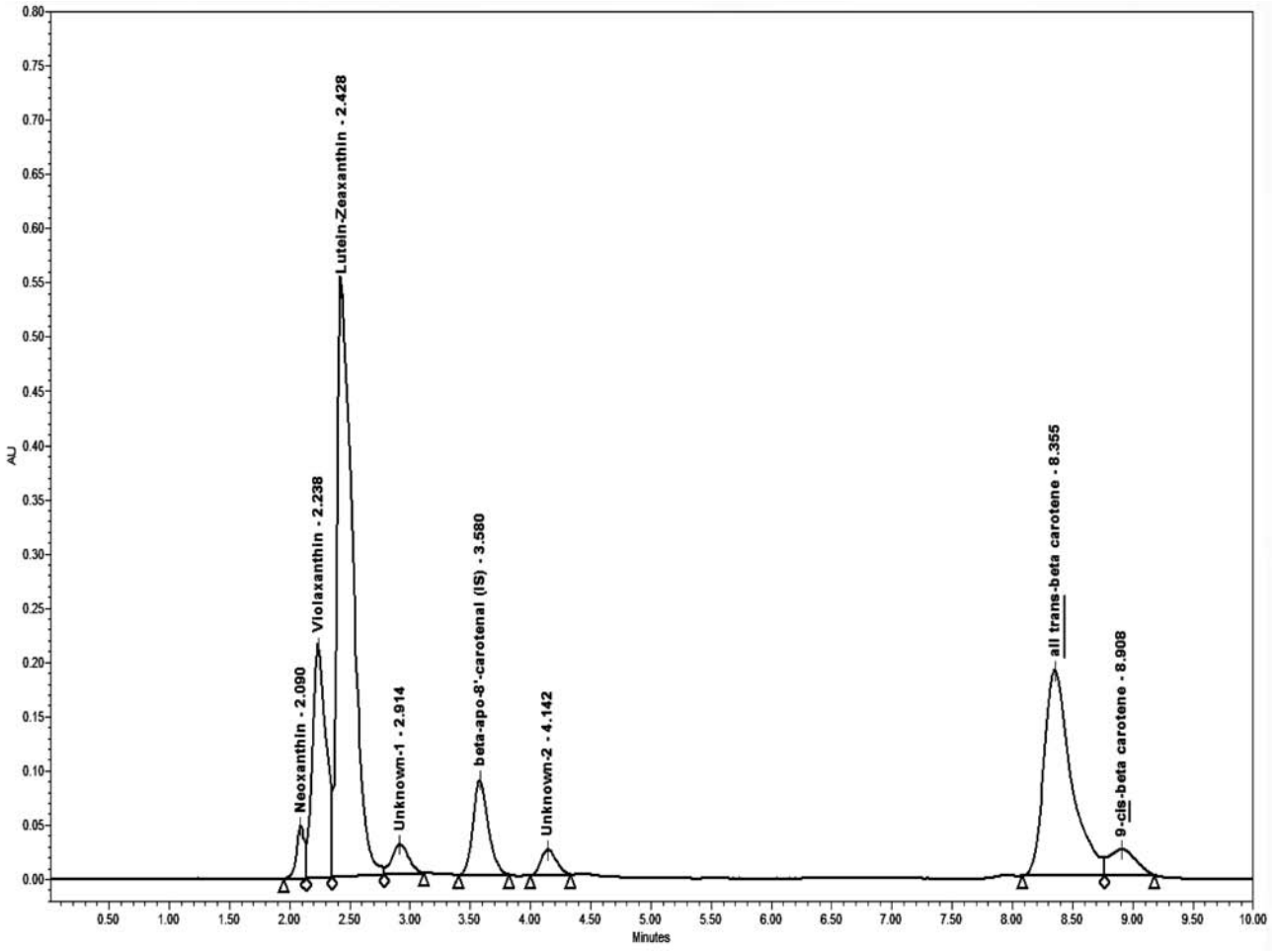

Figure 1. Carotenoids were separated by HPLC-DAD on a C18 column. Carotenoids were extracted from baby-leaf spinach leaves and monitored at 454 nm. β-Apo-8′-carotenal (internal standard) elutes at ~3.6 min.

Table 1. Results of Dosimetry for Spinach Irradiated at Target Doses of 0.5, 1.0, 1.5, and 2.0 kGy

target dose (kGy)	mean dose ± SEM (kGy)	minimum dose (kGy)	maximum dose (kGy)	dose uniformity ratio
0.5	0.54 ± 0.009	0.47	0.59	1.26
1.0	1.08 ± 0.016	0.93	1.17	1.26
1.5	1.60 ± 0.027	1.34	1.73	1.29
2.0	2.09 ± 0.039	1.92	2.36	1.23

violaxanthin, and vitamins B_9, E, and K. Ascorbic acid (total and free) concentrations, however, were linearly decreased with increased irradiation doses for both cultivars, and for 'Samish', dihydroascorbic acid linearly increased with increased irradiation doses.

Ascorbic Acid. Initial total ascorbic acid concentrations (total AsA) were ~14% higher in 'Samish' than in 'Lazio' leaves due mostly to a ~23% higher initial free ascorbic acid concentration (free AsA) in 'Samish' (**Table 3**). Both total and free AsA decreased with increasing doses of irradiation, with overall free AsA loss greater in 'Samish' (mean 41%) than in 'Lazio' (mean 26%). A loss in total and free AsA occurred with as little as 0.5 kGy for both 'Samish' and 'Lazio'; with 'Samish' having an additional loss with doses at or near 2.0 kGy. Relative decline in free AsA in 'Lazio' and 'Samish' followed increased doses of irradiation and coincided with relative increases in dihydroascorbic acid concentrations (dihydro-AsA) (**Table 3**). Initial dihydro-AsA concentrations were similar for 'Lazio' and 'Samish', but increased in N_2 versus air and with increased doses of irradiation, resulting in 'Samish' having mean overall increased dihydro-AsA of 23 versus 9% for 'Lazio'. An elevated level of dihydro-AsA, resulting from the oxidation of free AsA following irradiation, was previously described (*10*), and it is considered to be a reliable indicator of plant stress (*7*). In both 'Lazio' and 'Samish' free AsA likely declined due to exogenous stress occurring with a dose as low as 0.5 kGy.

Carotenoids. Detected levels of carotenoids in 'Lazio' and 'Samish' consisted of four known xanthophylls (lutein, neoxanthin, violaxanthin, zeaxanthin) with lutein and zeaxanthin coeluting, and one carotene (β-carotene), which eluted as two peaks, *all-trans* and 9-*cis*-β-carotene, and two minor unknown carotenoid peaks (**Figure 1**). The major caroteniods in 'Lazio' and 'Samish' spinach were lutein/zeaxanthin (47%) and β-carotene (33%), whereas known minor carotenoids were violaxanthin (18%) and neoxanthin (2%) (**Table 4**). Xanthophylls in 'Lazio' were variable in their response to increased doses of irradiation. Neoxanthin showed no effect to increasing doses of irradiation, whereas violaxanthin and lutein/zeaxanthin showed a difference with the highest dose (2.0 kGy). Xanthophylls in 'Samish' were relatively more sensitive than in 'Lazio'. All xanthophylls in 'Samish' were reduced following 2.0 kGy, whereas violaxanthin and lutein/zeaxanthin were initially reduced at 1.0−1.5 kGys. Although atmosphere effects were determined as nonsignificant (**Table 2**) lutein/zeaxanthin levels were reduced ~9% for both spinach cultivars, with ~6% loss under N_2 versus ~12% loss under air. Nitrogen atmosphere during irradiation is known to

Table 2. Summary Response of Vitamins,[a] on a Dry Weight Basis, and AsA on a Fresh Weight Basis to Bag Air or N_2 Atmosphere and Irradiation Level (0.0, 0.5, 1.0, 1.5, and 2.0 kGy)[b,c]

source	total AsA	free AsA	dihydro-AsA	β-carotene	lut/zea	neo	viola	vit B_9	vit E	vit K
				'Lazio'						
atmosphere	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
kGy	*	**	ns	ns	ns	ns	ns	ns	ns	ns
atmosphere × kGy	ns	ns	*	ns	ns	ns	ns	ns	ns	ns
polynomial	L*	L**	–	–	L*	Q*	–	C*	–	–
				'Samish'						
atmosphere	*	ns	**	ns	ns	ns	ns	ns	ns	ns
kGy	***	***	***	ns	ns	ns	ns	ns	ns	ns
atmosphere × kGy	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
polynomial	L**	L**	L**, Q*	–	Q*	Q**	–	–	–	L**

[a] AsA, ascorbic acid; vit B_9, folate; lut/zea, lutein/zeaxantin; neo, neoxanthin; vit E, α-tocopherol; viola, violaxanthin; vit K, phylloquinone. [b] Bag atmosphere and irradiation level were analyzed factorially. Stepwise sums of squares for the polynomial fit for irradiation level are linear (L), quadratic (Q), cubic (C), or no fit (–). [c] Significance: ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3. Effect of Increasing Doses of Irradiation under Air or N_2 Atmospheres on Percent Dry Weight, Total Ascorbic Acid (Total AsA), Free Ascorbic Acid (Free AsA), and Dihydroascorbic Acid (Dihydro-AsA) of Baby-Leaf Spinach from Two Cultivars

cultivar	atmosphere	irradiation dose (kGy)	dry wt (%)	total AsA (mg/100 g of FW)	free AsA (mg/100 g of FW)	dihydro-AsA (mg/100 g of FW)
Lazio	air	0.0	10.0 ± 0.4a[a]	66.3 ± 2.4a	41.6 ± 1.8a	24.4 ± 1.9a
Lazio	air	0.5	9.7 ± 0.3a	56.0 ± 2.4b	35.7 ± 2.8b	20.3 ± 2.6a
Lazio	air	1.0	10.1 ± 0.3a	56.4 ± 2.8b	32.2 ± 3.4b	24.1 ± 4.2a
Lazio	air	1.5	10.0 ± 0.3a	57.1 ± 3.6b	33.0 ± 5.1b	21.6 ± 3.7a
Lazio	air	2.0	10.0 ± 0.4a	56.2 ± 3.1b	32.7 ± 1.5b	23.5 ± 1.6a
$P \leq 0.01$						
Lazio	N_2	0.0	10.1 ± 0.1a	61.2 ± 2.5a	37.5 ± 2.6a	23.7 ± 2.3b
Lazio	N_2	0.5	10.2 ± 0.4a	55.8 ± 2.7b	35.9 ± 4.0a	20.8 ± 0.5b
Lazio	N_2	1.0	10.1 ± 0.2a	53.9 ± 5.2b	27.2 ± 3.7b	27.7 ± 4.0a
Lazio	N_2	1.5	10.1 ± 0.2a	55.1 ± 4.7b	26.3 ± 3.7b	27.9 ± 3.2a
Lazio	N_2	2.0	10.1 ± 0.2a	54.6 ± 2.3b	26.3 ± 2.0b	28.2 ± 2.2a
$P \leq 0.01$						
Samish	air	0.0	9.7 ± 0.2a	71.9 ± 1.8a	53.6 ± 2.7a	19.3 ± 1.5b
Samish	air	0.5	9.7 ± 0.2a	63.5 ± 4.7b	38.8 ± 3.7b	24.6 ± 1.3a
Samish	air	1.0	9.8 ± 0.3a	61.8 ± 1.7b	38.2 ± 3.2b	23.5 ± 1.5a
Samish	air	1.5	10.0 ± 0.3a	61.7 ± 1.7b	38.1 ± 1.0b	25.8 ± 2.2a
Samish	air	2.0	9.9 ± 0.3a	55.9 ± 1.0c	31.0 ± 0.5c	24.8 ± 0.7a
$P \leq 0.01$						
Samish	N_2	0.0	9.8 ± 0.2a	76.3 ± 2.5a	50.5 ± 2.3a	25.9 ± 2.2b
Samish	N_2	0.5	10.1 ± 0.2a	72.0 ± 3.5b	44.2 ± 2.7b	26.8 ± 1.5b
Samish	N_2	1.0	9.8 ± 0.3a	64.2 ± 1.5c	41.4 ± 2.7bc	34.9 ± 2.5a
Samish	N_2	1.5	10.0 ± 0.3a	64.8 ± 2.7c	31.8 ± 3.5c	33.1 ± 0.5a
Samish	N_2	2.0	10.0 ± 0.2a	63.7 ± 1.3c	29.2 ± 3.5c	34.5 ± 2.7a
$P \leq 0.01$						

[a] Means ± SD. Separations within a cultivar and within atmosphere across kGy followed by a different letter are significantly different by the two-way *t* test ($n = 24$).

reduce changes in carotenoids while reducing spoilage (*11*). β-Carotene concentrations did not show the probable nitrogen atmosphere benefit experienced by lutein/zeaxanthin. Also unlike the xanthophylls, β-carotene, depending on the cultivar, declined at 0.5−1.0 kGy and again at 1.5−2.0 kGy with an overall decline, depending on cultivar, of 12−17%.

α-Tocopherol. Of the many tocopherols found in plants, only α-tocopherol was found in our spinach, which was minimally affected by increased doses of irradiation and was not affected by package atmosphere (**Table 5**). 'Lazio' exhibited no change in the concentration of α-tocpherol even at 2.0 kGy, whereas 'Samish' was a little more sensitive to irradiation than 'Lazio', exhibiting a decline in α-tocopherol at 2.0 kGy. γ-Irradiation of pecan (*Carya illinoesnsis*) nut meats, a rich source of tocopherols, was also found to maintain their level of α-tocopherol even with irradiation doses as high as 3.0 kGy, but sensory attributes of the nut meats were adversely affected at this dose (*12*).

Folate. The vitamer 5-methyltetrahydrofolate (folate) is the most biologically important folate with respect to human health (*13*) and the vitamer assayed in this study. Folate was minimally affected by increased doses of irradiation and little affected by package atmosphere (**Table 5**). 'Lazio' under air exhibited a reduction in folate at 2.0 kGy as did 'Samish'. But under N_2 both 'Lazio' and 'Samish' exhibited a reduction with doses of 0.5−1.0 kGy. However, the overall percent loss in folate was less under N_2 than under air. The sensitivity to N_2 at lower doses of irradiation conflicts with the commercial use of N_2 storage to protect folates from oxidation in food products (*14*).

Table 4. Effect of Increasing Doses of Irradiation under Air or N_2 Atmospheres on Xanthophylls (Neoxanthin, Violaxanthin, and Lutein/Zeaxanthin) and Carotene (β-Carotene) of Baby-Leaf Spinach from Two Cultivars

cultivar	atmosphere	irradiation dose (kGy)	neoxanthin (μg/g of FW)	violaxanthin (μg/g of FW)	lutein/zeaxanthin (μg/g of FW)	β-carotene (μg/g of FW)
Lazio	air	0.0	3.7 ± 0.4a[a]	30.6 ± 2.0a	85.2 ± 2.6a	55.9 ± 1.7a
Lazio	air	0.5	3.5 ± 0.4a	30.8 ± 1.2a	84.6 ± 4.0a	54.9 ± 3.5a
Lazio	air	1.0	3.4 ± 0.4a	30.6 ± 1.5a	82.3 ± 4.0a	54.3 ± 3.0ab
Lazio	air	1.5	3.4 ± 0.6a	29.6 ± 2.0a	82.3 ± 3.2a	51.4 ± 2.5bc
Lazio	air	2.0	2.9 ± 0.7a	25.4 ± 1.7b	75.3 ± 3.3b	46.9 ± 3.0c
$P \leq 0.01$						
Lazio	N_2	0.0	3.5 ± 0.4a	29.9 ± 1.2a	85.6 ± 1.7a	59.1 ± 2.6a
Lazio	N_2	0.5	3.5 ± 0.4a	29.1 ± 3.0ab	85.0 ± 3.0a	60.2 ± 2.7a
Lazio	N_2	1.0	3.3 ± 0.7a	28.4 ± 2.0ab	84.3 ± 2.6ab	56.4 ± 3.0ab
Lazio	N_2	1.5	3.1 ± 0.5a	28.1 ± 2.0ab	83.6 ± 2.2ab	55.2 ± 3.0bc
Lazio	N_2	2.0	2.7 ± 0.6a	26.3 ± 3.0b	81.4 ± 2.7b	52.1 ± 2.5c
$P \leq 0.01$						
Samish	air	0.0	4.3 ± 0.2a	34.5 ± 1.1a	87.9 ± 3.0a	61.3 ± 2.7a
Samish	air	0.5	3.6 ± 0.9a	32.6 ± 3.0ab	83,9 ± 3.4a	57.7 ± 2.3ab
Samish	air	1.0	3.6 ± 0.8a	29.2 ± 3.0b	81.9 ± 3.1ab	57.4 ± 2.5b
Samish	air	1.5	3.5 ± 0.6ab	27.9 ± 3.0b	81.3 ± 2.3bc	54.2 ± 3.2bc
Samish	air	2.0	2.7 ± 0.4b	26.5 ± 2.0b	76.2 ± 3.0c	51.7 ± 2.5c
$P \leq 0.01$						
Samish	N_2	0.0	3.8 ± 0.2a	37.1 ± 2.7a	85.6 ± 2.5a	59.7 ± 3.7a
Samish	N_2	0.5	3.7 ± 0.7ab	32.6 ± 2.5b	84.0 ± 2.1ab	53.6 ± 2.3b
Samish	N_2	1.0	3.1 ± 0.3b	31.8 ± 3.6b	84.5 ± 4.0ab	52.9 ± 2.7bc
Samish	N_2	1.5	3.0 ± 0.4b	26.8 ± 2.2bc	80.9 ± 3.7bc	53.2 ± 2.5b
Samish	N_2	2.0	2.4 ± 0.3b	24.5 ± 2.0c	79.5 ± 4.7c	49.9 ± 4.2c
$P \leq 0.01$						

[a] Means ± SD. Separations within a cultivar and within atmosphere across kGy followed by a different letter are significantly different by the two-way *t* test ($n = 24$).

Table 5. Effect of Increasing Doses of Irradiation under Air or N_2 Atmospheres on α-Tocopherol, Folate, and Phylloquinone of Baby-Leaf Spinach from Two Cultivars

cultivar	atmosphere	irradiation dose (kGy)	α-tocopherol (μg/g of FW)	folate (μg/g of FW)	phylloquinone (μg/g of FW)
Lazio	air	0.0	13.2 ± 1.3a[a]	1.86 ± 0.27a	2.81 ± 0.21a
Lazio	air	0.5	12.6 ± 1.5a	1.60 ± 0.16a	2.75 ± 0.27ab
Lazio	air	1.0	12.5 ± 1.2a	1.54 ± 0.16a	2.56 ± 0.31ab
Lazio	air	1.5	12.5 ± 1.2a	1.61 ± 0.16a	2.48 ± 0.31ab
Lazio	air	2.0	12.0 ± 0.7a	1.32 ± 0.27b	2.23 ± 0.31b
$P \leq 0.01$					
Lazio	N_2	0.0	13.4 ± 1.5a	1.59 ± 0.14a	3.09 ± 0.23a
Lazio	N_2	0.5	12.8 ± 1.2a	1.25 ± 0.16b	2.66 ± 0.23ab
Lazio	N_2	1.0	12.7 ± 1.3a	1.23 ± 0.15b	2.48 ± 0.31ab
Lazio	N_2	1.5	12.4 ± 1.0a	1.17 ± 0.12b	2.62 ± 0.32ab
Lazio	N_2	2.0	12.4 ± 1.0a	1.24 ± 0.11b	2.43 ± 0.31b
$P \leq 0.01$					
Samish	air	0.0	13.5 ± 0.6a	1.71 ± 0.10a	3.29 ± 0.21a
Samish	air	0.5	13.2 ± 1.0a	1.62 ± 0.13a	2.93 ± 0.34a
Samish	air	1.0	13.0 ± 1.5ab	1.60 ± 0.29a	2.92 ± 0.32a
Samish	air	1.5	12.5 ± 1.7ab	1.50 ± 0.28ab	2.86 ± 0.34a
Samish	air	2.0	11.9 ± 1.7b	1.30 ± 0.27b	2.84 ± 0.33a
$P \leq 0.01$					
Samish	N_2	0.0	12.9 ± 0.7a	1.72 ± 0.16a	2.71 ± 0.22a
Samish	N_2	0.5	12.8 ± 0.8a	1.70 ± 0.28a	2.70 ± 0.24a
Samish	N_2	1.0	12.7 ± 1.0a	1.41 ± 0.17b	2.66 ± 0.24a
Samish	N_2	1.5	12.3 ± 1.0ab	1.38 ± 0.17b	2.25 ± 0.33a
Samish	N_2	2.0	11.4 ± 0.5b	1.33 ± 0.17b	2.23 ± 0.32a
$P \leq 0.01$					

[a] Means ± SD. Separations within a cultivar and within atmosphere across kGy followed by a different letter are significantly different by the two-way *t* test ($n = 24$).

Others have found that a significant radiation-induced loss of folate will occur in spinach under air, if irradiated with >2.5 kGy (*15*). However, if the same spinach was dehydrated, no loss in folate occurred at 2.5 kGy, thus demonstrating that folate is most sensitive to ionizing irradiation when in fresh tissues and storage atmosphere may be a confounding factor in its degradation.

Phylloquinone. Phylloquinone was reduced in 'Lazio' spinach at 2.0 kGy (**Table 5**), but was not affected in 'Samish'. There were

no differences between air and N_2 atmospheres for either cultivar. Of all the vitamins, phylloquinone is considered to be the least sensitive to γ-irradiation (*16*).

Conclusions. Of the eight spinach-leaf phytonutrients (vitamins) assayed, on either a fresh or dryweight (data not shown) basis, in this study four (α-tocopherol, folate, neoxanthin, and phylloquinone) relatively minor compounds exhibited, depending on cultivar, little or no change in concentration with increasing doses (0.0, 0.5, 1.0, 1.5, 2.0 kGy) of irradiation. AsA, on both a fresh and dryweight basis (data not shown), and lutein/zeaxanthin, violaxanthin, and β-carotene, on a fresh weight basis, were affected only at 2.0 kGy, and depending on cultivar, at 0.5 kGy and again at 1.5−2.0 kGy. Of the three most concentrated phytonutrients (AsA, lutein/zeaxanthin, and β-carotene), AsA was 8- and 12-fold more concentrated (μg/g of FW) than lutein/zeaxanthin and β-carotene, respectively, and was the most affected following increased doses of irradiation. Dihydro-AsA, an indicator of stress (*7*), coincidently increased as free AsA is oxidized by reactive oxygen species (ROS) (*17*). The principal role of free AsA in photosynthetic tissue is as an antioxidant, neutralizing ROS free radical compounds, particularly the most hazardous, hydrogen peroxide, which is favorably generated by ionizing irradiation in a hydrated systems, for example, leaves (*18*, *19*). Ascorbic acid, the only compound in our study to have up to a 42% loss in concentration following irradiation, may have been directly affected by ROS generated by irradiation. Variation in spinach cultivars' vitamin sensitivity to irradiation was observed, and variation in ROS scavenging ability is known (*7*). It is, therefore, likely that generation of ROS following irradiation may be more critical impact factor on AsA, β-carotene, and lutein/zeaxanthin concentrations than are direct doses of irradiation.

ACKNOWLEDGMENT

We thank Dr. D. J. Makus and Cruz Salinas (USDA-ARS, Weslaco, TX) for spinach production and Marc Villalobos (USDA-ARS, Weslaco, TX) for conducting irradiation treatments and quality analyses.

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Received for review January 13, 2010. Revised manuscript received March 4, 2010. Accepted March 10, 2010.