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Spinach Cultigen Variation for Tissue Carotenoid Concentrations Influences Human Serum Carotenoid Levels and Macular Pigment Optical Density Following a 12-Week Dietary Intervention

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Increasing intakes of carotenoid-rich plant foods can increase serum carotenoid concentrations and macular pigment optical density (MPOD) in most, but not all, individuals. Research objectives for this study were to (1) characterize tissue lutein (L) and β-carotene (BC) concentrations in carotenoid-rich spinach (*Spinacia oleracea* L.) cultigens and (2) determine serum carotenoid and MPOD responses in human subjects consuming spinach cultigens differing in tissue L and BC concentrations. Thirteen spinach cultigens were evaluated for carotenoid accumulations over two consecutive growing seasons. 'Springer' (8.4 and 6.5 mg/100 g of fresh mass for L and BC, respectively) and 'Spinner' (12.1 and 9.2 mg/100 g of fresh mass for L and BC, respectively) spinach cultigens were selected for a dietary intervention study and represented low- and high-L concentrations. The high-L ('Spinner') and low-L ('Springer') spinach treatment groups consisted of 10 subject volunteers ingesting five 50-g spinach servings/week during a 12-week intervention. Average serum L concentrations increased by 22% ($P = 0.07$) from baseline (0.233 μmol/L) to 12 weeks (0.297 μmol/L) for subjects consuming low-L spinach. Subjects consuming high-L spinach showed increases of 33% ($P = 0.04$) in serum L from baseline (0.202 μmol/L) to 12 weeks (0.300 μmol/L). Average MPOD did not change for the low-L treatment group; however, subjects in the high-L group demonstrated increases ($P = 0.02$) in MPOD at the 30′ eccentricity between baseline (0.343) and 12 weeks (0.374). This study demonstrates that serum carotenoid and MPOD are determined by L concentrations present in the spinach matrix. Results emphasize the role of cultigen selection among vegetable crops in determining phytochemical effects on human health.

KEYWORDS: **β-Carotene; germplasm; HPLC; human health; lutein; macular pigment**

INTRODUCTION

Carotenoids are a class of naturally occurring phytochemicals that demonstrate biological activity beyond normal health maintenance and nutrition. Because humans cannot synthesize carotenoids in vivo, ingestion of dietary sources is required to provide sufficient levels. The consumption of carotenoid-rich foods is associated with numerous health benefits. Increasing intakes of carotenoid-rich foods can increase serum carotenoids and macular pigment (MP) (*1*). Although increasing the number of servings consumed is one goal, enhancement efforts to increase carotenoid concentrations per serving could make a significant impact on health.

Carotenoids are C_{40} isoprenoid polyene compounds that form lipid-soluble yellow, orange, and red pigments in higher plants, bacteria, algae, and fungi (*2*, *3*). More than 600 carotenoids can be found in nature. They are divided into oxygenated xanthophylls such as lutein (L), zeaxanthin (Z), and violaxanthin and hydrocarbon carotenes such as β-carotene (BC), α-carotene, and lycopene (*2*). Among naturally occurring plant pigments,

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carotenoids are widely distributed, possess a high degree of structural diversity, and demonstrate large variations in biological function (*4*, *5*).

One of the most important physiological functions of carotenoids in human nutrition is as vitamin A precursors. Pro-vitamin A carotenoids support maintenance of healthy epithelial cell differentiation, normal reproductive performance, and visual functions. Pro-vitamin A carotenoids (BC and α-carotene) and non-pro-vitamin A carotenoids (L and Z) can also function as free radical scavengers, enhance the immune response, and protect eye tissues and may suppress cancer development (*6*). L and Z are two of seven carotenoids found in human blood; however, they are typically the only carotenoids present in the eye (*7*). In the retina, L and Z are chiefly responsible for the yellow pigmentation referred to as MP (*8*). These yellow pigments are critical to photoprotective functions, and low levels of MP may be related to retinal damage (*9*, *10*).

The bioavailability of carotenoids from plant foods is highly variable and is influenced by the types and structures of carotenoids present in the food, the rate of release from the food matrix, the amount consumed and absorption in the intestinal tract, transportation within the lipoprotein fractions, biochemical conversions, tissue-specific depositions, and the nutritional status of the ingesting subject (*2*, *11*, *12*). After release from the food matrix, carotenoids are assimilated and oriented into lipid micelles before uptake by intestinal mucosal cells. After release to the enterocyte, carotenoids are incorporated into chylomicrons and eventually delivered to the liver through the blood stream. Carotenoid compounds can remain in the liver or be transferred to low-density (LDL) or high-density (HDL) lipoproteins before eventual tissue-specific deposition (*2*). Increasing intakes of carotenoid-rich foods or carotenoid dietary supplements will increase serum carotenoid concentrations, macular pigment optical density (MPOD), and tissue carotenoid accumulations in some, but not all, subjects (*1*, *6*, *13*−*15*). Several studies have demonstrated increased serum carotenoids and MPOD from spinach (*Spinacia oleracea* L.) consumption (*1*, *13*, *16*). Moreover, food frequency questionnaire studies indicate high fruit and vegetable intake correlates with high serum carotenoids and high MPOD (*17*, *18*). Because carotenoid bioavailability and MPOD may be modified through increased intake of carotenoid-rich foods, our research objectives were to (1) characterize tissue L and BC accumulations among carotenoid-rich spinach cultigens, (2) determine serum carotenoid responses in subjects consuming spinach cultigens differing in tissue L and BC concentrations, and (3) determine MPOD responses in subjects consuming spinach cultigens differing in tissue L and BC concentrations.

MATERIALS AND METHODS

Plant Culture for Spinach Genetic Screen. Thirteen spinach cultigens including commercial cultigens ('Hector', 'Indian Summer', 'Space', 'Spinner', 'Springer', and 'Tyee' from Johnny's Selected Seed, Winslow, ME; 'Melody' and 'Olympia' from Harris Seeds, Rochester, NY; 'Polydane' and 'Unipak 12' from Stokes Seeds, Buffalo, NY) and USDA-ARS accessions ('PI 606707', 'NSL 6082', and 'NSL 6084' from USDA-ARS-NCRPIS, Regional Plant Introduction Station, Ames, IA) were evaluated for carotenoid accumulations over two consecutive growing seasons. Spinach seeds were sown in artificial media (Promix BX; Premier Horticulture, Dorval, Quebec, Canada) on April 16, 2002, and April 28, 2003. The medium was supplied with bottom heat (23 °C), and plants were greenhouse grown (22 °C day/14 °C night set points) for ∼4 weeks under natural photoperiods (latitude 43° 09′ N, Durham, NH). Peter's 20N−6.9P−16.6K water-soluble fertilizer (Scotts Co., Marysville, OH) was applied every 5 days at a rate of 200 mg/L.

Spinach plants were transplanted into the field at the Woodman Horticulture Farm, Durham, NH, on May 16, 2002, and May 28, 2003. Fertilizer was applied to plots of a Charlton sandy loam 1 week prior to transplanting, and cultigens were grown according to New England Cooperative Extension guidelines for spinach (*19*). Each cultigen was planted in a plot consisting of two rows of eight plants each at the recommend spacing of 16 cm within rows and 31 cm between rows. Plots were replicated four times in a randomized complete block design for each year of evaluation. Irrigation was supplied during plant growth to ensure plants received a total of 2.5 cm of water per week. Nitrogen (as NH_4NO_3) was applied as a side-dressing at a rate of 33.6 kg of N/ha on June 3, 2002, and June 15, 2003. Plants were harvested on June 14, 2002, and June 27, 2003. FromDuring May 16 to June 14, 2002, the average daily temperature was 15 °C (with no days reaching temperatures >30 °C); average daily photosynthetically active radiation (PAR) was 555 μmol/m^2/s, and total rainfall was 12 cm (University of New Hampshire weather station, Durham, NH). From May 28 to June 27, 2003, the average daily temperature was 17 °C (with 5 days reaching temperatures >30 °C), average daily PAR was 439 μmol/m^2/s, and total rainfall was 8 cm. At harvest, 10 uniform plants per replicate were collected and combined for carotenoid analysis. Tissues were lyophilized for 48 h (model 6 L FreeZone; LabConCo, Kansas City, MO) and stored at −80 °C prior to extraction.

Carotenoid Determination for Spinach Genetic Screen. *Tissue Extraction.* Freeze-dried tissues samples were ground with dry ice in a kitchen grinder (Handy Chopper Plus, HC 3000, Household Products Inc., Shelton, CT). Pigments were extracted and separated according to previously published methods (*20*, *21*). A 0.1-g subsample was placed into a Potter−Elvehjem tissue grinder tube (Kontes, Vineland, NJ) and hydrated with 0.8 mL of deionized water. The sample was placed in a 40 °C water bath for 20 min. After hydration, 0.8 mL of the internal standard, ethyl-β-apo-8′-carotenoate (Sigma Chemical Co., St. Louis, MO), and 2.5 mL of tetrahydrofuran (THF), stabilized with 25 mg/L 2,6-di-*tert*-butyl-4-methoxyphenol (BHT), were added. The sample was homogenized in the tube with ∼25 insertions with a Potter−Elvehjem tissue grinder pestle attached to a drill press (Sears, Roebuck and Co., Hoffman Estates, IL) at 540 rpm. The sample tube was kept immersed in ice. The tube was placed into a clinical centrifuge for 3 min at 500g_n. The supernatant was removed with a Pasteur pipet, placed into a conical 15-mL test tube, capped, and held on ice. The sediment was resuspended in 2.0 mL of THF and homogenized with ∼25 insertions of the grinding pestle. The tube was centrifuged for 3 min at 500g_n, and the supernatant was collected and combined with the first extracted supernatant. The extraction procedure was repeated twice more until the supernatant was colorless. The sediment was discarded, combined supernatants were placed in a 40 °C water bath, and the volume was reduced to 0.5 mL under a stream of nitrogen (model N-EVAP 111; Organomatic, Inc., Berlin, MA). After the addition of 2.5 mL of MeOH and 2.0 mL of THF to the 0.5-mL sample, the solution was vortexed and filtered through a 0.2-μm polytetrafluoroethylene filter (model Econofilter PTFE 25/20; Agilent Technologies, Wilmington, DE).

HPLC Analysis. A HPLC unit with photodiode array detector (Agilent 1100, Agilent Technologies, Palo Alto, CA) was used for pigment separation and detection (**Figure 1**). All samples were analyzed for carotenoid compounds using a Vydac RP-C_{18} 5.0 μm 250 × 4.6 mm column (model 201TP54, Phenomenex, Torrance, CA) fitted with a 4 × 3.0 mm, 7.0-μm guard column compartment. The column was maintained at 16 °C using a thermostatic column compartment (Agilent Technologies, Palo Alto, CA). The mobile phase eluents were (A) 75% acetronitrile, 20% methanol, 5% hexane, 0.05% BHT, and 0.013% triethylamine (TEA) (v/v) and (B) 50% acetonitrile, 25% THF, 25% hexane, and 0.013% TEA (v/v). The flow rate was 0.7 mL/min, and the gradient was 100% eluent A for 30 min, 50% A and 50% B for 2 min, 100% B for 2 min, and 50% A and 50% B for 2 min. The mobile phase was returned to 100% A for 10 min prior to the next sample injection. Separated carotenoid compounds from a 20.0-μL injection loop were detected at 452 (carotenoids and internal standard), 652 (chlorophyll *b*), and 665 (chlorophyll *a*) nm, with data collected and integrated using 1100 HPLC ChemStation software (Agilent Technologies, Palo Alto, CA). Peak assignment for individual pigments was performed by comparing retention times and line spectra obtained from

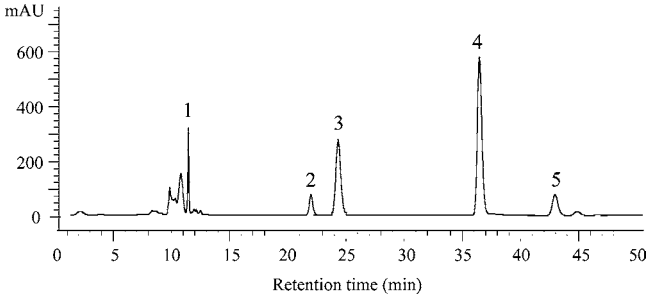

Figure 1. Representative HPLC chromatogram of carotenoid pigment separation in spinach using a C_{18} column. Identified peaks and retention times are as follows: (1) lutein (11.39 min); (2) ethyl-β-8′-apo-carotenoate internal standard (22.97 min); (3) chlorophyll *b* (24.57 min); (4) chlorophyll *a* (37.04 min); and (5) β-carotene (42.66 min). HPLC conditions are described in the text. Absorbances at 452, 652, and 665 nm were combined to depict carotenoid and chlorophyll contents.

Table 1. Descriptive Characteristics for Subject Volunteers Partitioned into the Spinach Treatment Groups of (1) No Spinach (Control Treatment), (2) High-Lutein Spinach Group, or (3) Low-Lutein Spinach Treatment Groups in the 12-Week Dietary Intervention Study

	no-spinach group	high-lutein spinach group	low-lutein spinach group
participants	$n = 10$	$n = 10$	$n = 10$
sex			
males	4	4	3
females	6	6	7
mean age (years)	39.1	39.2	35.0
mean BMI[a]	24.4	25.6	23.7

[a] Body mass index (BMI) calculated as weight/height (kg/m^2).

photodiode array detection using external standards (lutein and zeaxanthin from Carotenature, Lupsingen, Switzerland; β-carotene, chlorophyll *a*, and chlorophyll *b* from Sigma Chemical Co.). External standard concentrations were determined spectrophotometrically using the following $E^{1\%}_{1cm}$ values: β-carotene, 2592 in hexane, $\lambda_{max} = 452$ nm; lutein, 2550 in ethanol, $\lambda_{max} = 445$ nm; zeaxanthin, 2540 in ethanol, $\lambda_{max} = 452$ nm (*22*). A spinach standard reference material (Slurried Spinach 2385, National Institute of Science and Technology, Gaithersburg, MD) was used for method validation. Recovery of the internal standard was used to correct for sample losses during extraction, preparation, and filtration.

Subject Recruitment for Dietary Intervention. The use of human subjects in this study was approved by the University of New Hampshire Institutional Review Board and complied with the Helsinki Declaration. In brief, subjects were recruited by word of mouth and advertisements in local papers. Individuals were excluded if they were current smokers or had gastrointestinal, heart, or eye disease. Thirty individuals, between 21 and 60 years of age, agreed to participate in the feeding trial. After baseline measures of serum carotenoids and MPOD, they were randomly assigned to (1) no-spinach (control treatment), (2) high-L spinach, or (3) low-L spinach treatment groups. Descriptive characteristics of the participants partitioned in the treatment groups appear in **Table 1**.

Spinach Culture and Recipe Preparation for Dietary Intervention. 'Spinner' and 'Springer' spinach cultigens were selected for the dietary intervention study on the basis of differences in L concentrations between the two cultigens. 'Spinner' spinach represented a high-L-accumulating cultigen, whereas 'Springer' spinach represented a low-lutein-accumulating cultigen (**Table 2**). Both cultigens were direct seeded at the Woodman Horticulture Farm every 6 days for three planting dates beginning August 21, 2003. Fertilization, plant spacing, and plant culture were as described above for the genetic screen. Spinach plants were harvested on October 6, 2003, and October 13, 2003. From August 21 to October 13, 2003, the average daily temperature was 16.1 °C (with no days reaching temperatures >30.0 °C); PAR was 386.1 μmol/m^2/s. High-L and low-L spinach plants were kept separate, and leaf tissues were triple-rinsed and wilted in a commercial kitchen on each harvest date. Both cultigens were partitioned into 50-g subsamples for unadorned spinach and incorporated into different prepared spinach dishes (spinach enchiladas, spinach and cheese stuffed shells, creamed spinach, curried lentil and spinach soup, and roasted red peppers and spinach) for the feeding intervention. All prepared dishes contained a fat source for carotenoid absorption and provided 50-g spinach serving sizes. Prepared spinach dishes and unadorned spinach were vacuum sealed and stored at −4 °C. The high-L ('Spinner') spinach group averaged 12.1 and 9.2 mg/100 g of fresh mass for L and BC, respectively. Carotenoid values for the low-L ('Springer') spinach group averaged 8.4 and 6.5 mg/100 g of fresh mass for L and BC, respectively. Subject volunteers received two to three prepared spinach dishes per week; the other servings were unadorned frozen spinach, which the subjects may or may not have incorporated into another dish. Subjects were instructed to consume one serving per day for 5 days each week and to consume the unadorned servings with a fat source. Control treatment subjects received no spinach. All subjects were instructed not to modify their existing diets, with the exception of treatment inclusion.

Table 2. Mean Values[a] of Lutein and β-Carotene for 13 *S. oleracea* L. Cultigens over Two Growing Seasons (2002 and 2003) in Durham, NH (Latitude 43° 09′ N)

	pigment concn (mg/100 g of fresh mass)			
	lutein		β-carotene	
cultigen	2002	2003	2002	2003
PI 606707	11.15 ± 0.06	8.93 ± 1.34	8.65 ± 0.61	5.95 ± 0.95
Hector	9.89 ± 2.18	8.43 ± 1.38	7.74 ± 1.90	5.76 ± 0.96
Indian Summer	11.32 ± 1.96	6.49 ± 1.23	8.01 ± 0.95	4.11 ± 1.27
Melody	10.61 ± 1.83	7.65 ± 1.91	8.10 ± 2.15	5.37 ± 1.19
NSL 6082	10.63 ± 2.39	8.62 ± 1.45	6.57 ± 0.79	4.73 ± 0.86
NSL 6084	8.73 ± 1.22	6.91 ± 0.82	7.56 ± 1.11	4.98 ± 0.78
Olympia	8.95 ± 1.35	7.76 ± 1.35	7.42 ± 1.35	5.52 ± 0.46
Polydane	8.69 ± 0.75	7.97 ± 0.43	7.13 ± 0.05	5.58 ± 0.16
Space	9.28 ± 0.82	7.87 ± 0.95	7.06 ± 0.73	5.07 ± 0.21
Spinner	12.98 ± 0.26	6.79 ± 0.40	10.94 ± 0.52	4.67 ± 0.72
Springer	8.11 ± 0.93	6.63 ± 1.20	7.05 ± 0.90	4.48 ± 0.15
Tyee	10.62 ± 1.64	9.57 ± 1.06	8.24 ± 1.35	5.94 ± 0.75
Unipak 12	9.49 ± 0.81	9.26 ± 1.59	8.16 ± 1.26	5.90 ± 1.25
mean	10.02	8.02	7.89	5.34
$LSD_{0.05}$[b]	2.41	2.53	2.07	1.76
$LSD_{0.05}$[c]	1.69		1.38	

[a] Composition of leaf samples from 4 replications, 10 plants each, ± standard deviation. [b] Composition of leaf samples from 4 replications, 10 plants each, ± standard deviation. LSD for differences between cultivar means within year. [c] LSD for differences between cultivar means between years.

Bioavailability Assessments. *Serum Carotenoids.* Carotenoid bioavailability was assessed in serum and MPOD responses at initial subject recruitment (baseline) and at the end of the 12 week dietary intervention. Serum L, Z, and BC were measured according to previously published methods (*18*, *23*, *24*). Blood was drawn from fasting subjects (10−12 h) into serum separation Vacutainer tubes (366511SS, Gel and Clot Activator gray and red tiger tops, Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1650g_n for 15 min at −4 °C (model TJ-6; Beckman-Coulter, Fullerton, CA). Samples were stored at −80 °C prior to HPLC analysis. The HPLC system, column conditions, and data analysis were similar to those of methods outlined above for spinach tissue pigments. For serum samples, HPLC mobile phase eluents were (A) 100% MeOH (buffered with 0.1% ammonium acetate) and (B) MeOH/methylene chloride (80:20, v/v; buffered with 0.1% ammonium acetate). The flow rate was 1.5 mL/min for the mobile phase gradient transitioning from 100% A to 100% B over 10 min. The mobile phase was then held at 100% B for 15 min before returning to 100% A for 10 min prior to the next injection. Eluted carotenoid compounds from a 20.0-μL injection loop were detected at 452 nm. Serum carotenoids were quantified by assessing chromatogram peak

areas and calibrating them against known amounts of external standards (National Institute of Science and Technology, Gaithersburg, MD).

Measurement of Macular Pigment Optical Density. Heterochromatic flicker photometry was used to measure MPOD in each subject's right eye (*25*). In this technique, the difference in short-wavelength sensitivity between a foveal locus, which is screened by macular pigment, and a parafoveal locus, which is not screened by macular pigment, is used to estimate MPOD. For the foveal measure, subjects viewed a centrally fixated 1° disk. The parafoveal measure was performed with a 2° disk centered at 7° nasal eccentricity. The targets were composed of two modulating lights, a 570-nm reference light (1.7 log Tds) and a 460-nm test light, superimposed on a 6°, 470-nm background (1.5 log Tds). Subjects adjusted the radiance of the test light until the flickering target appeared to be stable (i.e., ceased to flicker). They performed this task six times for both targets. MPOD was calculated by subtracting the mean log energy of the six parafoveal measures from the mean log energy of the six foveal measures. A Macular Metrics (Rehoboth, MA) densitometer was used to produce the test stimuli (*10*).

Statistical Analysis. Spinach tissue data were analyzed by the GLM procedures of SAS (Cary, NC) with cultigen means separated by least significant difference (LSD) within and between growing seasons. Paired *t* tests ($\alpha = 0.05$) were used to measure differences in serum carotenoid values and MPOD between baseline and week 12 for the subject treatment groups.

RESULTS

Genetic Variation in Spinach Carotenoid Accumulation. Lutein concentrations differed among spinach cultigens ($F = 1.89$; $P = 0.05$) and year of cultivation ($F = 38.35$; $P < 0.001$) and for the interaction between cultigen and year ($F = 2.08$; $P = 0.04$). Total L concentrations among the spinach cultigens ranged from a high of 12.98 mg ('Spinner') to a low of 6.49 mg/100 g of fresh mass ('Indian Summer') (**Table 2**). Lutein concentration was significantly higher in 2002 than in 2003 ($P < 0.001$). During 2002, L concentration ranged from 12.98 ('Spinner') to 8.11 ('Springer') mg/100 g of fresh mass. During 2003, 'Tyee' accumulated the highest concentration of L (9.57 mg/100 g of fresh mass), whereas 'Indian Summer' accumulated the lowest concentrations of L (6.49 mg/100 g of fresh mass) (**Table 2**).

β-Carotene concentrations differed between years of cultivation ($F = 92.45$; $P < 0.001$), but not among spinach cultigens ($F = 1.54$; $P = 0.14$) or for the interaction between cultigen and year ($F = 1.68$; $P = 0.10$). Total BC concentrations among the spinach cultigens ranged from a high of 10.94 mg ('Spinner') to a low of 4.11 mg/100 g of fresh mass ('Indian Summer') (**Table 2**). β-Carotene concentrations were significantly higher in 2002 than in 2003 ($P < 0.001$). During 2002, BC concentrations ranged from 10.94 ('Spinner') to 6.57 ('NSL 6082') mg/100 g of fresh mass. During 2003, 'PI 606707' accumulated the highest concentration of BC (5.95 mg/100 g of fresh mass), whereas 'Indian Summer' accumulated the lowest concentrations of BC (4.11 mg/100 g of fresh mass) (**Table 2**).

When calculated on a dry mass basis, L concentrations differed among spinach cultigens ($F = 1.98$; $P = 0.04$) and years of cultivation ($F = 25.64$; $P < 0.001$) and for the interaction between cultigen and year ($F = 2.28$; $P = 0.02$). Lutein concentrations expressed on a dry mass basis ranged from 1.01 mg ('Tyee') to 0.66 mg/g of dry mass ('Olympia' and 'Spinner') (**Table 3**). β-Carotene concentrations expressed on a dry mass basis were not affected by spinach cultigen ($F = 1.53$; $P = 0.22$), year of cultivation ($F = 0.69$; $P = 0.75$), or the interaction between cultigen and year ($F = 1.48$; $P = 0.16$). 'Spinner' accumulated the highest concentrations of BC in 2002 (0.65 mg/g of dry mass) and the lowest accumulations in 2003 (0.45 mg/g of dry mass) (**Table 3**).

Table 3. Mean Values[a] of Lutein and β-Carotene for 13 *S. oleracea* L. cultigens over two growing seasons (2002 and 2003) in Durham, NH (Latitude 43° 09′ N)

	pigment concn (mg/g of dry mass)			
	lutein		β-carotene	
cultigen	2002	2003	2002	2003
PI 606707	0.72 ± 0.07	0.87 ± 0.05	0.56 ± 0.10	0.58 ± 0.03
Hector	0.70 ± 0.07	0.88 ± 0.06	0.54 ± 0.08	0.60 ± 0.04
Indian Summer	0.80 ± 0.04	0.77 ± 0.12	0.57 ± 0.07	0.49 ± 0.14
Melody	0.76 ± 0.12	0.81 ± 0.15	0.58 ± 0.15	0.57 ± 0.10
NSL 6082	0.77 ± 0.13	0.85 ± 0.22	0.56 ± 0.13	0.47 ± 0.16
NSL 6084	0.67 ± 0.09	0.69 ± 0.02	0.57 ± 0.05	0.50 ± 0.02
Olympia	0.66 ± 0.07	0.82 ± 0.13	0.55 ± 0.06	0.59 ± 0.04
Polydane	0.69 ± 0.06	0.91 ± 0.08	0.57 ± 0.01	0.64 ± 0.04
Space	0.71 ± 0.09	0.87 ± 0.10	0.54 ± 0.08	0.56 ± 0.02
Spinner	0.78 ± 0.08	0.66 ± 0.03	0.65 ± 0.06	0.45 ± 0.02
Springer	0.71 ± 0.08	0.75 ± 0.11	0.55 ± 0.02	0.51 ± 0.01
Tyee	0.76 ± 0.08	1.01 ± 0.08	0.59 ± 0.07	0.63 ± 0.04
Unipak 12	0.68 ± 0.05	0.86 ± 0.06	0.59 ± 0.09	0.56 ± 0.06
mean	0.72	0.83	0.57	0.55
$LSD_{0.05}$[b]	0.13	0.17	0.13	0.11
$LSD_{0.05}$[c]	0.10		0.09	

[a] Composition of leaf samples from 4 replications, 10 plants each, ± standard deviation. [b] LSD for differences between cultivar means within year. [c] LSD for differences between cultivar means between years.

Serum Carotenoid Bioavailability. Average serum L concentrations increased by 22% ($P = 0.07$) from baseline assessment (0.233 μmol/L) to the end of the 12-week intervention (0.297 μmol/L) for subjects consuming the low-L spinach. Subjects consuming the high-L spinach demonstrated increases of 33% ($P = 0.038$) in serum L concentrations from baseline (0.202 μmol/L) to the end of the 12-week intervention (0.301 μmol/L). Serum Z levels did not differ between baseline and week 12 assessment in subjects consuming either low-L ($P = 0.542$) or high-L ($P = 0.267$) spinach. Average L plus Z serum concentrations increased by 11% from baseline (0.238 μmol/L) to week 12 of the intervention (0.311 μmol/L), but did not differ between assessments ($P = 0.266$) for subjects consuming the low-L spinach. Subjects consuming the high-L spinach demonstrated increases of 32% ($P = 0.044$) in serum L plus Z concentrations from baseline (0.228 μmol/L) to the end of the 12-week intervention (0.334 μmol/L). Although not significant, serum BC concentrations decreased by 15% ($P = 0.482$) and by 22% ($P = 0.524$) from baseline to the end of the 12-week intervention for subjects consuming the low-L and high-L spinach, respectively. There were no differences in serum carotenoid concentrations between the baseline and 12-week assessments for subjects receiving no spinach in the control group (**Table 4**).

Vision Assessment and Carotenoid Bioavailability. Average MPOD at 30′ eccentricity decreased by 2% and did not differ ($P = 0.482$) between baseline assessment (0.354) and the end of the 12-week intervention (0.346) for subjects consuming the low-L spinach. However, subjects consuming the high-L spinach demonstrated an increase of 8% ($P = 0.021$) in average MPOD at 30′ eccentricity between baseline assessment (0.343) and the end of the 12-week intervention (0.374), indicating increases in the protective MP in the retina. There were no differences in average MPOD at 30′ eccentricity between the baseline and 12-week assessments for subjects receiving no spinach in the control group (**Table 4**).

Table 4. Mean Values for Serum Carotenoids and Macular Pigment Optical Density (MPOD) at 30-min Locus (30′) in Subject Volunteers at a Baseline Visit and at the Conclusion of a 12-Week Dietary Intervention during Which Subject Treatments Consisted of No Spinach as a Control Group and 50-g Samples of either High-Lutein ['Spinner' (Tissue Lutein and β-Carotene at 12.1 and 9.2 mg/100 g of Fresh Mass, Respectively)] or Low-Lutein ['Springer' (Tissue Lutein and β-Carotene at 8.4 and 6.5 mg/100 g of Fresh Mass, Respectively)] Whole Spinach Five Times per Week[a]

bioavailability assessment	baseline	week 12	*t* ratio	$P > t$[b]
No-Spinach Group				
serum carotenoids (μmol/L)				
lutein	0.257 ± 0.052	0.269 ± 0.055	0.708	0.497
zeaxanthin	0.035 ± 0.005	0.031 ± 0.005	–0.732	0.483
lutein + zeaxanthin	0.292 ± 0.056	0.300 ± 0.059	0.387	0.709
β-carotene	0.395 ± 0.103	0.398 ± 0.113	0.073	0.943
MPOD				
30′	0.314 ± 0.038	0.306 ± 0.035	–0.351	0.734
High-Lutein Spinach Group[c]				
serum carotenoids (μmol/L)				
lutein	0.202 ± 0.026	0.301 ± 0.051	2.427	0.038
zeaxanthin	0.025 ± 0.004	0.034 ± 0.008	1.184	0.267
lutein + zeaxanthin	0.228 ± 0.029	0.334 ± 0.057	2.335	0.044
β-carotene	0.335 ± 0.125	0.260 ± 0.068	–0.664	0.524
MPOD				
30′	0.343 0.043	0.374 ± 0.039	2.779	0.021
Low-Lutein Spinach Group[c]				
serum carotenoids (μmol/L)				
lutein	0.233 ± 0.039	0.297 ± 0.062	2.041	0.072
zeaxanthin	0.047 ± 0.030	0.030 ± 0.006	–0.634	0.542
lutein + zeaxanthin	0.278 ± 0.066	0.311 ± 0.068	1.187	0.266
β-carotene	0.329 ± 0.079	0.281 ± 0.062	–0.734	0.482
MPOD				
30′	0.354 ± 0.044	0.346 ± 0.039	–0.483	0.640

[a] Values represent means ± standard error . [b] *t*-statistic degrees of freedom = 9, $\alpha = 0.05$. [c] Spinach cultigens were field-grown from August 21 to October 13, 2003, in Durham, NH (latitude 43° 09′ N).

DISCUSSION

The major finding in the current study was the demonstration that serum L concentrations and MPOD were affected by different L concentrations in spinach. Several studies have shown positive responses in serum carotenoids and MPOD through increased consumption of spinach, as well as other vegetable crops (*1*, *13*, *18*). To our knowledge, this is the first study to measure serum carotenoid and MPOD responses after consumption of two distinct spinach cultigens differing in tissue L concentrations. Subjects consuming spinach ('Spinner') with higher L concentrations showed significant increases in serum L, serum L plus Z, and MPOD at 30′ from baseline to the end of the 12-week intervention. Differences in subject responses can be attributed to differences in tissue L concentrations between the two spinach cultigens, assuming similar matrix, release of carotenoids from the matrix, absorption in the intestinal micelles, and translocation and deposition of L.

Carotenoid concentrations in vegetable crops appear to be shaped by a plant species' physiological, genetic, and biochemical attributes, as well as environmental growth factors such as light, temperature, and fertility (*20*, *26*−*28*). Significant genetic variation for carotenoid accumulations within vegetable crop species exists for carrot (*Daucus carota* L. var. *sativa*) (*29*), corn (*Zea mays* L.) (*30*), kale (*Brassica oleracea* L. var. *acephala* D.C.) (*20*, *26*, *31*), lettuce (*Lactuca* species) (*32*), potato (*Solanum tuberosum* subsp. *tuberosum* L.) (*33*), pepper (*Capsicum* species) (*34*, *35*), and soybean (*Glycine max* L.) (*36*). Significant genetic variation for L and BC accumulation in the leaf tissues of spinach is reported in the current study. Differences of >2-fold for carotenoid pigment accumulation among spinach cultigens are similar to previous reports for carotenoid variation among other leafy vegetable crop species (**Tables 2** and **3**).

Environmental growing conditions can influence carotenoid accumulations in vegetable crops. In the current study, carotenoid concentrations in the spinach cultigens differed significantly between years of cultivations, most likely due to differences in growing temperatures between 2002 and 2003. Carotenoid concentrations can fluctuate in response to environmental manipulations, with responses differing among plant species. Previous reports demonstrated the influence of growing air temperature on carotenoid accumulations in both kale and spinach. Spinach carotenoid concentrations were shown to decrease linearly from 11.2 to 7.3 mg/100 g of fresh mass as the growing air temperature increased from 10 to 25 °C (*37*). In the current study, air temperatures in 2003 were warmer (especially late in the growing season) than in 2002, resulting in significantly less carotenoid concentrations during the 2003 evaluation. Manipulation of cultural growing conditions and time of harvest would therefore be influential on carotenoid concentrations in vegetable crops.

All carotenoids exhibit *cis*−*trans* isomerization, and both isomeric groups can be found in vegetable crops (**Figure 2**) (*21*, *38*−*40*). *All-trans* carotenoids in plants are susceptible to photoisomerization, thermal isomerization, and chemical isomerization (*41*). Human blood contains mostly *all-trans* carotenoids; however, some carotenoids can be found as high as 50% in the *cis* form (*42*). Because of their lipophilic nature, biotic or abiotic activities that expose carotenoid molecules to potential oxidation, degradation, or isomerization will ultimately influence carotenoid biochemistry and bioavailability. Food-processing activities, such as thermal processing and mincing, result in changes to carotenoid chemistry, which usually increases bioavailability through increased release of bound carotenoids from the food matrix (*12*, *39*, *43*, *44*). Absorption of carotenoids in humans is mostly passive but can be protein-mediated through specific scavenger receptor proteins in normal lipid digestive pathways (*45*). Protein- or membrane-bound carotenoids must first be released from plant tissues and dissolved into hydrophobic domains (oils, fats, or bulk lipid emulsions). Due to their hydrophobic nature, carotenoids in the mostly aqueous environment in plant tissues must be transferred to bulk lipids or intestinal micelles (*11*). For this reason, the presence of dietary fat in the small intestine increases carotenoid absorption (*2*). Recent studies provide evidence of increased carotenoid absorption when plant foods are consumed with accompanying dietary lipids (*46*, *47*).

Carotenoid bioavailability is easily assessed in serum at various time intervals following ingestion of whole foods or supplements. Recent studies demonstrated *in vitro* Caco-2 cells can also accurately predict carotenoid bioavailability from both supplements and whole foods (*15*, *48*, *49*). Serum carotenoid concentrations typically increased after ingestion of carotenoids from whole food or monomolecular supplements (*6*, *12*, *13*, *43*, *46*, *47*, *50*). *In vitro* and *in vivo* studies showed that carotenoid bioavailability is influenced by carotenoid source (whole food vs. supplement), degree of processing, interactions with other ingested carotenoid compounds, amount of dietary fat, transit time in the intestine, and the nutritional status of the human subjects (*11*). In the current study, the 'Spinner' spinach treatment group showed significant increases in serum L concentrations at the end of the 12-week intervention. During

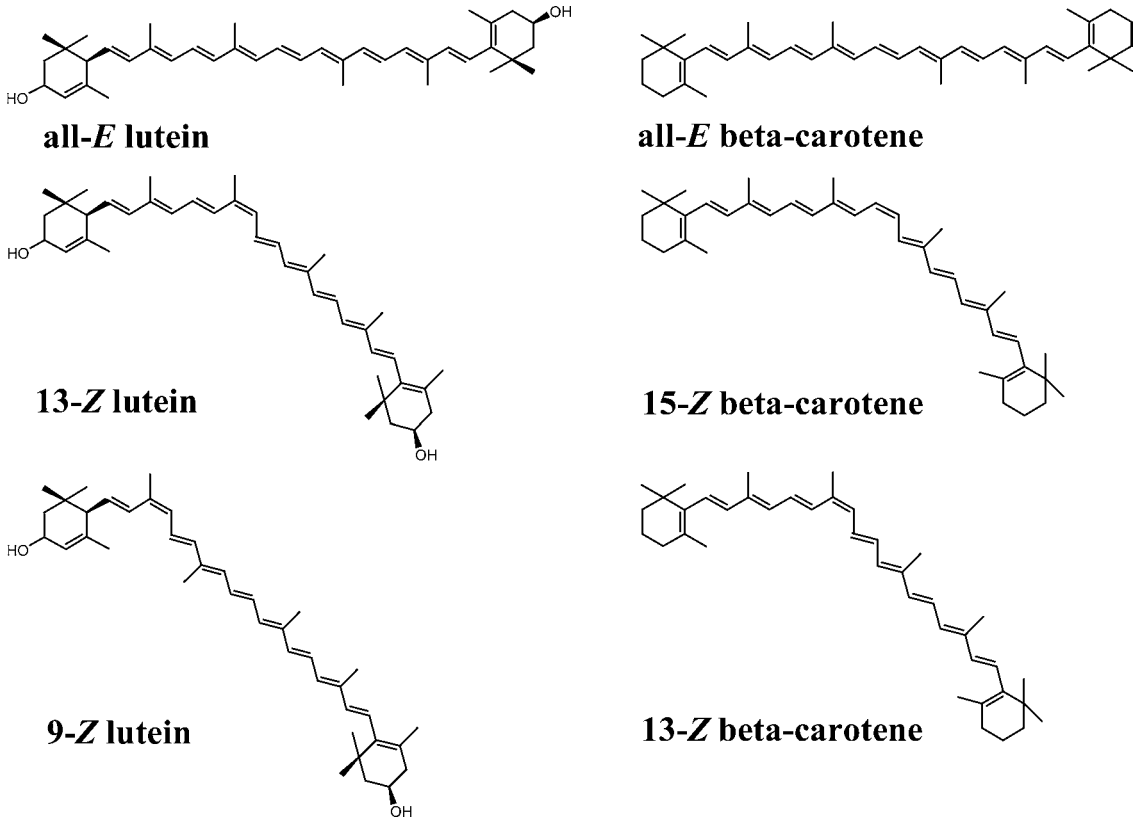

Figure 2. Structures of *all-E* and (*-Z*) isomers of lutein and β-carotene typically found in vegetable crops. The occurrence and properties of carotenoid isomers in food crops may influence intestinal absorption and bioavailability.

the intervention, both groups demonstrated a decrease in serum BC concentrations, even though the spinach cultigens were high in tissue BC levels. Johnson et al. (*1*) reported a similar nonresponse in serum BC concentrations in subjects consuming spinach with tissue BC levels similar to the current study. These combined results may demonstrate that L is more bioavailable from the spinach matrix than BC or that other dietary changes may have influenced BC.

One possible explanation for differences in bioavailability between L and BC from the spinach matrix may come from differences in molecular orientation in plant membranes between carotenes and xanthophylls. Hydrophobic interactions and lack of polar end groups localize carotenes, such as BC, within the hydrophobic core of biomembranes at several different orientations. The positions of xanthophyll carotenoids (L) are oriented to span the entire membrane, allowing for the positioning of polar groups outside the hydrophobic core or in the polar headgroup region of the membrane (*51*). Differences in molecule positioning may be expected to affect the release of carotene and xanthophyll carotenoids from the matrix of plant tissues and, thus, affect bioavailability.

Macular pigment can be modulated through increased ingestion of carotenoids and other antioxidants, high fruit and vegetable consumption, maintaining a normal body mass index, and a history of no tobacco use. Many of these same factors are also associated with a decreased risk of developing age-related macular degeneration, which suggests there may be a causal relationship (*52*, *53*). Epidemiological evidence is inconsistent regarding potential relationships among diet, genetics, and environment and the risk of developing age-related eye diseases. For this reason, a direct correlation between MP levels and development of macular diseases has not been established, although strong associative relationships are reported (*9*, *54*).

Conclusions from this study support earlier research showing the ability to modify both serum carotenoid concentrations and MPOD through increased consumption of carotenoid-rich plant foods. Unique to our study is the demonstration that serum carotenoid concentrations and MPOD were affected by the concentrations of spinach tissue L. These results show the direct nutritional impact of carotenoid enhancement in plant tissues and emphasize the importance of phytochemical enhancement efforts in fruit and vegetable crops. Increasing plant tissue carotenoid concentrations can be accomplished through cultural management techniques or simple cultigen selection. Changing dietary habits is difficult for most people. Results from this study indicate that nutritional benefits can be achieved by consuming spinach with higher carotenoid concentrations, which may be better than simply consuming greater quantities of spinach with lower carotenoid concentrations.

ABBREVIATIONS USED

BC, β-carotene; BHT, 2,6-di-*tert*-butyl-4-methoxyphenol; HPLC, high-performance liquid chromatography; L, lutein; LSD, least significant difference; MP, macular pigment; MeOH, methanol; MPOD, macular pigment optical density; PAR, photosynthetically active radiation; TEA, triethylamine; THF, tetrahydrofuran; USDA, U.S. Department of Agriculture; Z, zeaxanthin.

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