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Determination of Major Carotenoids in a Few Indian Leafy Vegetables by High-Performance Liquid Chromatography

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Leafy vegetables [*Basella rubra* L., *Peucedanum sowa* Roxb., *Moringa oleifera* Lam., *Trigonella foenum-graecum* L., *Spinacia oleracea* L., *Sesbania grandiflora* (L.) Poir., and *Raphanus sativus* L.] that are commonly used by the rural population in India were evaluated in terms of their main carotenoid pattern. The extracted carotenoids were purified by open column chromatography (OCC) on a neutral alumina column to verify their identity by their characteristic UV−visible absorption spectra. Reverse-phase high-performance liquid chromatography (HPLC) on a C_{18} column with UV−visible photodiode array detection under isocratic conditions was used for quantification of isolated carotenoids. Acetonitrile/methanol/dichloromethane (60:20:20 v/v/v) containing 0.1% ammonium acetate was used as a mobile phase. The major carotenoids identified by both methods were lutein, β-carotene, violaxanthin, neoxanthin, and zeaxanthin. Among the carotenoids identified, lutein and β-carotene levels were found to be higher in these leafy vegetables. Results show that *P. sowa* and *S. oleracea* are rich sources of lutein (77−92 mg/100 g of dry wt) and β-carotene (36−44 mg/100 g of dry wt) compared with other leafy vegetables. The purity of carotenoids eluted by OCC was clarified by HPLC, and they were found to be 92% ± 3% for neoxanthin, 94% ± 2% for violaxanthin, 97% ± 2% for lutein and zeaxanthin, and 90% ± 3% for β-carotene. It could be recommended to use *P. sowa* and *S. oleracea* as rich sources of lutein and β-carotene for health benefits. The OCC method proposed is relatively simple and provides purified carotenoids for feeding trials.

KEYWORDS: Leafy vegetables; carotenoids; chromatography

INTRODUCTION

Leafy and green vegetables are most commonly consumed and are accessible throughout the year in India and other parts of the world. They are also rich in bioactive molecules such as carotenoids and polyphenols with health promoting potential (*1*). The majority of health benefits of carotenoids are due to their provitamin A and antioxidant properties (*2*). Micozi and Moon (*3*) have reported an inverse relationship between the consumption of carotene-rich dark green vegetables and risk of incidence of human cancer. Epidemiological studies have demonstrated that consumption of carotenoid-rich fruits and vegetables is associated with lower incidence of cancer (*4*), cardiovascular disease (*5*), age-related macular degeneration (AMD), and cataract formation (*6*).

The composition of carotenoids in leafy vegetables markedly varies with variety, part of the plant and its degree of maturity at harvest, and climatic or geographic conditions as well as cultivation and postharvest handling practices. Chen and Chen (*7*) reported 100 ± 8 μg of β-carotene, 78 ± 7 μg of lutein, 60 ± 5 μg of violaxanthin, and 50 ± 5 μg of neoxanthin per gram of wet weight in *Ipomoea aquatica*, while Wills and Rangga (*8*) have reported much lower content of these carotenoids in the same species. To overcome such variations, data on the composition of carotenoids in green leafy vegetables grown in different geographic regions is very much essential. This would help in educating and encouraging the rural communities to consume more carotenoid-rich vegetables to derive maximum health benefits in addition to overcoming vitamin A malnutrition (*9, 10*) and AMD (*11*). Sincere attempts at generating accurate qualitative and quantitative data on carotenoids have resulted in the development of analytical techniques for the separation, identification, and quantification of these compounds (*12*, *13*). Analytical techniques for the isolation and development of high-performance liquid chromatography (HPLC) conditions for the separation of polar and nonpolar carotenoids from leafy vegetables are the need of the day. Many reports on the extraction and separation of various classes of carotenoids by HPLC technique are available (*12, 14*). However, the separation and quantification of major carotenoids from green leafy

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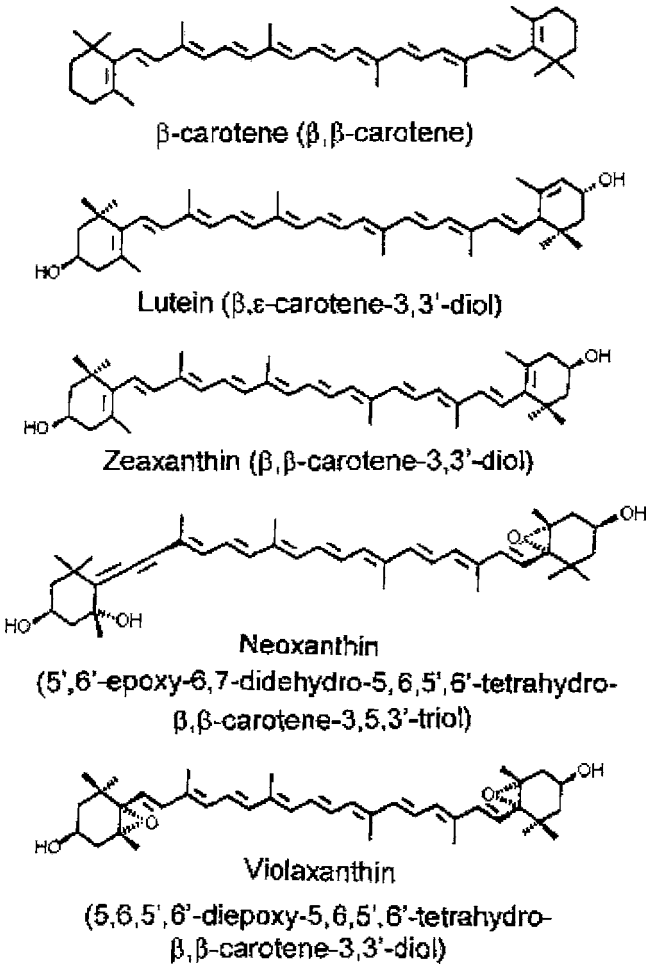

Figure 1. Chemical structures of major carotenoids present in leafy vegetables.

vegetables has not received as much attention as it deserves. Hence, the development of rapid column chromatographic and HPLC methods, which can separate carotenoids within a reasonable time, is warranted in order to assess the level of different carotenoids predominant in green leafy vegetables. Chemical structures and systematic names of major carotenoids found in green leafy vegetables are shown in **Figure 1** (*15*).

Among available research reports on HPLC analysis of carotenoids, the work of Kimura and Rodriguez-Amaya (*15*), Weller and Breithaupt (*16*), Hart and Scott (*17*), and Baskaran et al. (*18*), who isolated carotenoids from various leafy vegetables and from biological samples, is noteworthy. Similarly, Khachik et al. (*19*) have reported predominant carotenoids and carotenoid fatty acid esters in extracts from several varieties of squash by column chromatographic techniques. The present study was carried out to determine the composition of carotenoids in several green leafy vegetables by HPLC. Further, individual carotenoids were purified by open column chromatography (OCC) in order to obtain carotenoids for feeding experiments in rats to assess the influence of dietary phospholipids on their bioavailability (*20*). These techniques could be employed to separate both polar (neoxanthin, violaxanthin, lutein, zeaxanthin, and β-cryptoxanthin) and nonpolar carotenoids (β-carotene and α-carotene). Furthermore, the outcome of the present study shall help in selecting leafy vegetables rich in suitable carotenoids for consumption or supplementation for specific health purposes.

Table 1. Botanical, Common, and Local Names of Leafy Vegetables Used in This Study

botanical name	common name	local name
Basella rubra L.	red spinach	basale
Peucedanum sowa Roxb.	Indian dill	sabsige
Moringa oleifera Lam.	drumstick	nuggeyele
Trigonella foenum-graecum L.	fenugreek	menthya
Spinacia oleracea L.	spinach	palak
Sesbania grandiflora (L.) Poir.	agathi	agase
Raphanus sativus L.	radish	mullangi

MATERIALS AND METHODS

Samples. Freshly harvested green leafy vegetables used in this study were obtained from local farms on the day of analysis in the morning (24−26 °C) and immediately brought to the laboratory. For each leafy vegetable, three sample lots collected from three different farms were used for the analysis. The botanical, common, and local names of these leafy vegetables are given in **Table 1**. The leafy portions without stems were separated and used for the extraction of carotenoids. To obtain a homogeneous sample, a known amount (50−100 g) of each leafy vegetable was washed with deionized water, drained, and ground for 5 min in a blender and processed immediately.

Chemicals. Standard *all-trans-β*-carotene (98%), lutein (99%), α-tocopherol, and chlorophylls *a* and *b* were purchased from Sigma−Aldrich (St. Louis, MO). Neoxanthin (95%), violaxanthin (98%), and zeaxanthin (98%) were kindly donated by Dr. Akhihiko Nagao (National Food Research Institute, Tsukuba, Japan). Methanol, acetonitrile, dichloromethane, and hexane were of HPLC grade, and ethanol and ammonium acetate were purchased from S.D. Fine Chemicals (Mumbai, India). Neutral alumina (particle size 70−230 mesh) was from HiMedia, (Mumbai, India).

Extraction of Carotenoids. Fresh green leafy vegetables (50 g each) were ground well in a blender along with sodium sulfate (5 g) and 2 mM α-tocopherol in methanol (100 μL/g). Carotenoids were extracted from the ground leafy vegetables in ice-cold acetone and the extraction was repeated until the samples became colorless (total volume 400 mL). The crude extract (50 mL) was mixed and shaken well with 100 mL of hexane and the upper layer was separated by use of a separatory funnel. The extraction was repeated three times (total volume 250 mL). The pooled hexane extract was dried over anhydrous sodium sulfate (20 g) and filtered through Whatman no. 1 filter paper. The filtrate was evaporated to dryness in a rotary evaporator (Buchi) at 30−35 °C and redissolved in a known volume of hexane. An aliquot (100 μL) of extract was dried under a stream of nitrogen and the residue was redissolved in 1 mL of acetonitrile/methanol/dichloromethane (60:20:20 v/v/v). Samples were analyzed by HPLC. An aliquot of hexane extract was applied to OCC in order to purify individual carotenoids for further studies. Handling, homogenization, and extraction were carried out at 4 °C under dim yellow light to minimize photoisomerization and oxidation of carotenoids.

HPLC Analysis. The carotenoids were separated on a SGE C-18 (ODS) column, 25 cm × 4.6 mm i.d., 5 μm, 120A0 (SGE Co., India). Acetonitrile/methanol/dichloromethane (60:20:20 v/v/v) containing 0.1% ammonium acetate was used as a mobile phase for the separation of carotenoids. Samples were injected (20 μL) for HPLC analysis and an isocratic condition was maintained at a flow rate of 1 mL/min. All the carotenoids were monitored at 450 nm with UV−visible detector (Shimadzu, Japan). The peak identities and λ_{max} values of these compounds were confirmed by their retention times and characteristic spectra of standard chromatograms, recorded with a Shimadzu model LC-10Avp series equipped with SPD-10AVP detector. They were quantified from their peak areas in relation to the respective reference standards.

Column Chromatography. Carotenoids were separated by open column chromatography (OCC, 20 cm × 1.5 cm) on neutral alumina (particle size 70−230 mesh) by use of specific solvent systems modified from the procedures previously described by Rodriguez-Amaya (*21*) and Kimura and Rodriguez-Amaya (*22*). In brief, an aliquot of the acetone extract was evaporated to dryness under nitrogen, redissolved

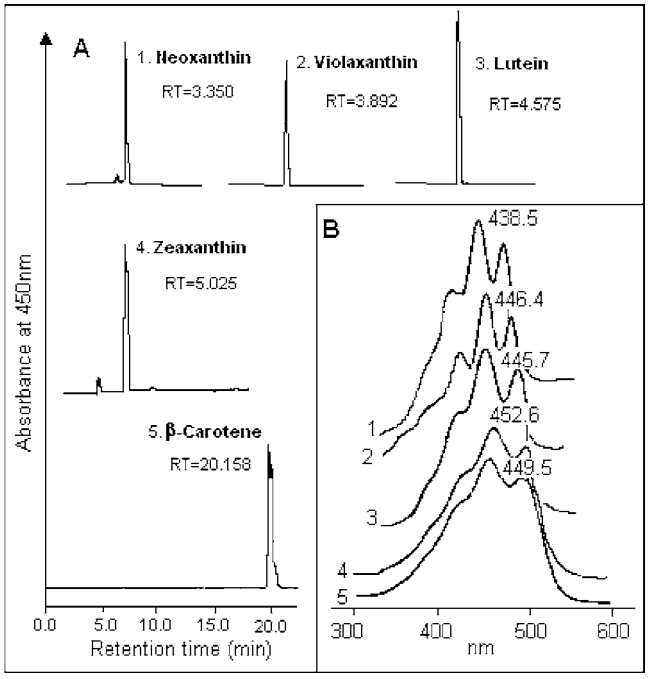

Figure 2. HPLC profile of standard carotenoids (**A**) and absorption spectra (**B**) of carotenoids purified from leafy vegetables studied: (1) neoxanthin, (2) violaxanthin, (3) lutein, (4) zeaxanthin, and (5) β-carotene.

Table 2. Reported and Observed UV–Visible Absorption Maxima (λ_{max}) for Carotenoids from Leafy Vegetables

carotenoids	λ_{max}[a]	λ_{max}[b,c]
neoxanthin	438.5	436
violaxanthin	446.4	440
β-carotene	449.5	448
lutein	445.7	446
zeaxanthin	452.6	452[d]

[a] Obtained in the present study. [b] Reported by [c] Chen and Chen (*7*) and [d] Emenhiser et al. (*27*).

in a known volume of hexane, and applied onto the alumina column. The β-carotene was eluted with hexane, the lutein and zeaxanthin fraction was eluted with methanol/dichloromethane (1:1 v/v), and the fractions rich in violaxanthin and neoxanthin were eluted with ethyl acetate/hexane (5:5 v/v) and ethyl acetate/hexane (1:9 v/v), respectively. The purity of an individual elute was analyzed by HPLC against respective reference standards. The peak identity, their respective spectra, and absorption maxima (λ_{max}) of carotenoids were confirmed by HPLC.

RESULTS AND DISCUSSION

Major pigments in green leafy vegetables used in the present study consist of three classes of carotenoids. In the order of chromatographic elution on a C_{18} column, these are xanthophylls (oxygenated carotenoids), chlorophylls, and hydrocarbon carotenoids and these pigments were separated within 22 min (**Figure 3**). The detectable xanthophylls comprising neoxanthin (peak 1), violaxanthin (peak 2), lutein (peak 3), and zeaxanthin (peak 4) and then the chlorophylls (peaks 5 and 6) and β-carotene (peak 7) as the hydrocarbon carotenoid were eluted under isocratic conditions and confirmed by their retention time and absorption spectra of respective reference standards (**Figure 2**). Kimura and Rodriguez-Amaya (*15*) isolated these carotenoids from lettuce by HPLC under gradient condition, which required

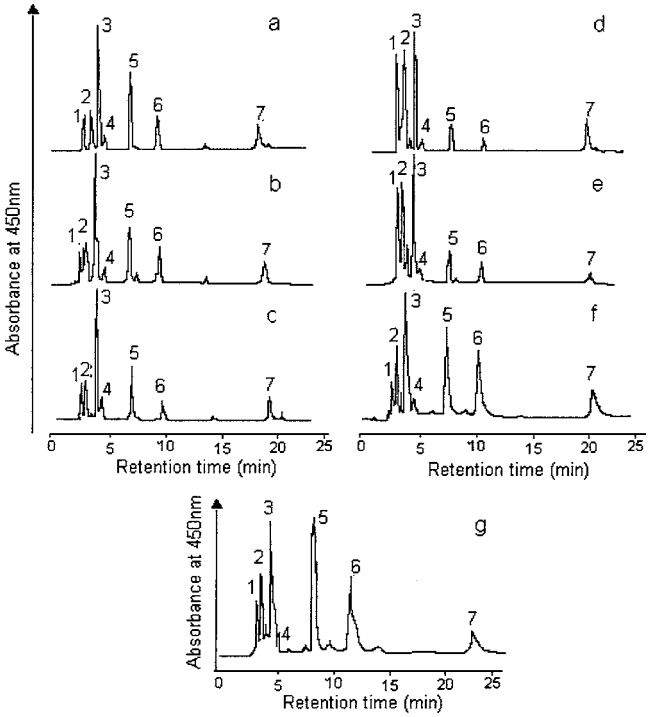

Figure 3. HPLC profile of carotenoids isolated from leafy vegetables: (a) *Basella rubra*, (b) *Peucedanum sowa*, (c) *Moringa oleifera*, (d) *Trigonella foenum-graecum*, (e) *Spinacia oleracea*, (f) *Sesbania grandifora*, and (g) *Raphanus sativus*. Peaks: (1) neoxanthin, (2) violaxanthin, (3) lutein, (4) zeaxanthin, (5) chlorophyll *b*, (6) chlorophyll *a*, and (7) *trans*-β-carotene. HPLC conditions are described in the text.

50 min to separate them, whereas this study reports that carotenoids in leafy vegetables were separated within 22 min under isocratic conditions.

The chromatographic profiles of the carotenoids in leafy vegetables studied were found to be almost identical (**Figure 3**). The major differences among these leafy vegetables appear to be the levels at which the various carotenoids are present. For example, the chromatogram from the extract of *B. rubra* (**Figure 3A**) shows the presence of both hydrocarbon and xanthophylls carotenoids; however, their relative concentrations were found to be different compared with other leafy vegetables studied (**Table 3**). For example, β-carotene levels (milligrams per 100 grams of dry weight) in *P. sowa* (44.53 ± 5.7) and *S. oleracea* (36.53 ± 6.4) were significantly ($p < 0.05$) higher compared to those of *B. rubra* (32.42 ± 3.3), *M. oleifera* (22.89 ± 6.8), *T. foenum-graecum* (12.13 ± 4.1), *S. grandiflora* (13.28 ± 3.2), and *R. sativus* (11.20 ± 1.9). These differences may be related to species variations and various environmental factors. Chen and Chen (*7*) have reported that factors such as species, part of the plant, degree of maturity at harvest, cultivation, and postharvest handling practices influence the carotenoid levels. Khachik et al. (*12*), Hart and Scott (*17*), and Tee and Lim (*23*) reported β-carotene levels in *Spinacia oleracea*, *Spinacia pumila*, and *Amaranthus viridis* of 3.39, 3.17, and 6.71 mg/100 g edible portion, respectively.

Neoxanthin has been isolated, which is the most common allene in the green leaves, and has also been associated with violaxanthin and lutein. Earlier studies have reported a transformation in the end group of one of these carotenoids may perhaps be responsible for the formation of the allenic end group (*24*). The differences (percentage) between neoxanthin, violaxanthin, and lutein among leafy vegetables studied may be

Table 3. Carotenoid Composition of Green Leafy Vegetables[a]

leafy vegetables	neoxanthin	violaxanthin	lutein	zeaxanthin	total xanthophylls[b]	β-carotene	total carotenoids[c]
B. rubra L.	12.01 ± 1.3	6.94 ± 0.7	67.94 ± 7.4	2.25 ± 0.4	89.14 ± 9.8	32.42 ± 3.3	121.56 ± 13.1
P. sowa Roxb.	8.85 ± 1.1	17.74 ± 2.0	92.99 ± 2.2	2.25 ± 0.6	121.83 ± 5.8	44.53 ± 5.7	166.36 ± 11.5
M. oleifera Lam.	9.60 ± 0.6	18.30 ± 3.2	50.40 ± 0.8	4.13 ± 0.7	82.43 ± 5.3	22.89 ± 6.8	104.56 ± 12.1
T. foenum-graecum L.	3.32 ± 0.5	33.26 ± 2.7	59.60 ± 8.3	0.95 ± 0.2	97.13 ± 11.7	12.13 ± 4.1	109.26 ± 15.8
S. oleracea L.	58.00 ± 4.6	65.00 ± 9.3	77.58 ± 6.6	1.51 ± 0.4	202.09 ± 20.9	36.53 ± 6.4	238.62 ± 27.3
S. grandiflora (L.) Poir.	1.857 ± 0.2	4.33 ± 0.8	16.90 ± 3.7	0.57 ± 0.7	23.65 ± 5.4	13.28 ± 3.2	36.93 ± 8.6
R. sativus L.	3.30 ± 0.3	5.40 ± 0.4	22.30 ± 8.0	0.75 ± 0.1	31.75 ± 8.8	11.20 ± 1.9	42.95 ± 10.7

[a] Data presented as milligrams per 100 grams of dry weight. [b] Total xanthophylls = neoxanthin + violaxanthin + lutein + zeaxanthin. [c] Total carotenoids = total xanthophylls + hydrocarbon carotenoid.

attributed to these biosynthetic transformations (*25*, *26*). The level of total xanthophylls separated in these leafy vegetables is about 5−10 times greater than that of the hydrocarbon carotene. Xanthophyll values reported here for spinach are about 4−6 times greater than those reported by Khachik et al. (*12*). Wills and Rangga (*8*) also reported higher levels of xanthophylls in leafy vegetables *A. tuberosum*, *A. tricolor*, and *B. chinensis* than those of hydrocarbon carotenoids. Further, in general the hydroxylation of α-carotene is known to be responsible for the formation of 3-hydroxy cyclic carotenoids and epoxy carotenoids; the absence of α-carotene in the leafy vegetables studied here may therefore be related to the complete conversion of these compounds to lutein. This may be a reason for the higher content of lutein (ranged between 16 and 92 mg/100 g of dry wt) in all the leafy vegetables studied (**Table 3**).

The purity of column-purified individual carotenoids on neutral alumina ranged between 92% ± 3% for neoxanthin, 94% ± 2% for violaxanthin, 97% ± 2% for lutein and zeaxanthin, and 90% ± 3% for β-carotene. β-Cryptoxanthin and α-carotene were not amenable for quantitation since they were below the detectable limit (0.1 pmol). These values were almost consistent with the results of Kimura and Rodriguez-Amaya (*22*), who have purified carotenoids from leafy vegetable using OCC on MgO:hyflosupercel (1:1). They have reported the purity as 91−97% for neoxanthin, 95−98% for violaxanthin, 92−96% for lutein, and 90−97% for β-carotene. The absorption maxima (λ_{max}) for xanthophylls and the hydrocarbon carotenoid (**Table 2**) isolated from leafy vegetables were found to be comparable with the reported values in the literature (*7*, *27*). They have reported λ_{max} for lutein (446 nm), β-carotene (448 nm), violaxanthin (440 nm), and neoxanthin (436 nm) eluted at 440 nm and for zeaxanthin (452 nm).

In conclusion, this study shows that the leafy vegetables analyzed here are found to contain higher levels of xanthophylls than β-carotene. The data generated on the composition of carotenoids in green leafy vegetables could be helpful to suggest using these leafy vegetables as a part of daily meal to overcome health problems such as vitamin A deficiency and AMD.

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LITERATURE CITED

(1) Moller, S. M.; Jacques, P. F.; Blumberg, J. B. The potential role of dietary xanthophylls in cataract and age-related macular degeneration. *J. Am. Coll. Nutr*. **2000**, *19*, 522S−527S.

(2) Zanutto, M. E.; Jordao, A. A.; Vannucchi, H. Improvement in vitamin status with consumption of dark green vegetables: a bioavailability study in rabbits. *Nutr. Res*. **2003**, *23*, 271−278.

(3) Micozi, M.; Moon, T. *Nutrition and cancer prevention: The role of micronutrients*. Marcel Dekker: New York, 1988.

(4) Bowman, P. E.; Mobarhan, S. Evidence from cancer intervention and biomarker studies and the development of biochemical markers. *Am. J. Clin. Nutr*. **1995**, *62*, 1403S−1409S.

(5) Krichevsky, S. B. β-Carotene, carotenoids and the prevention of coronary heart disease. *J. Nutr*. **1999**, *129*, 5−8.

(6) Landrum, J. T.; Bone, R. A. Lutein, zeaxanthin, and the macula pigment. *Arch. Biochem. Biophys*. **2001**, *385*, 28−40.

(7) Chen, B. H.; Chen, Y. Y. Determination of carotenoids and chlorophylls in water convolvulus (*Ipomea aquatica*) by liquid chromatography. *Food Chem*. **1992**, *45*, 129−134.

(8) Wills, R. B. H.; Rangga, A. Determination of carotenoids in Chinese vegetables. *Food Chem*. **1996**, *56*, 451−455.

(9) Suber, A. F.; Heimendinger, J.; Patterson, B. H; Krebs-Smith, S. M.; Pivonka, E.; Kessler, R. Fruit and vegetable intake in the U.S.: the baseline survey of the 5-a-Day for Better Health Program. *Am. J. Health Promotion* **1995**, *9*, 352−360.

(10) USDA-NCC Carotenoid Database for U.S. Foods. 1998. www.nal.usda.gov/fnic/foodcomp/Data/car98/car98.html.

(11) Mares-Perman, J. A.; Fisher, A.; Klein, R.; Palta, M.; Block, G.; Millen, A. E.; Wright, J. D. Lutein and zeaxanthin in the diet and serum and their relation to age-related maculopathy in the Third National Health and Nutrition Examination Survey. *Am. J. Epidemiol*. **2001**, *153*, 424−432.

(12) Khachik, F.; Beecher, G. R.; Whittaker, N. F. Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extract of several green vegetables by liquid chromatography. *J. Agric. Food Chem*. **1986**, *34*, 603−616.

(13) Khachik, F.; Beecher, G. R. Application of a C-45-β-carotene as an internal standard for the quantification of carotenoids in yellow/orange vegetables by liquid chromatography. *J. Agric. Food Chem*. **1987**, *35*, 732−738.

(14) Edelenbos, M.; Christensen, L. P.; Grevsen, K. HPLC Determination of chlorophyll and carotenoid pigments in processed green pea cultivars (*Pisum sativum* L.). *J. Agric. Food Chem*. **2001**, *49*, 4768−4774.

(15) Kimura, M.; Rodriguez-Amaya, D. B. Carotenoid composition of hydroponic leafy vegetables. *J. Agric. Food Chem*. **2003**, *51*, 2603−2607.

(16) Weller, P.; Breithaupt, D. Identification and quantification of zeaxanthin esters in plants using liquid chromatography−mass spectrometry *J. Agric. Food Chem*. **2003**, *51*, 7044−7049.

(17) Hart, D. J.; Scott, K. J. Development and evaluation of an HPLC method for the analysis of carotenoids in food, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the U.K. *Food Chem*. **1995**, *54*, 101−111.

(18) Baskaran, V.; Sugawara, T.; Nagao, A. Phospholipids affect the intestinal absorption of carotenoids in mice. *Lipids* **2003**, *38*, 705−711.

(19) Khachik, F.; Steck, A.; Pfander, H. Isolation and structural elucidation of (13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-lutein from marigold flowers, kale and human plasma. *J. Agric. Food Chem*. **1999**, *47*, 455−461.

(20) Raju, M.; Lakshminarayana, R.; Krishnakantha, T. P.; Baskaran, V. Influence of phospholipids on β-carotene absorption and conversion into vitamin A in rats. *J. Nutr. Sci. Vitaminol*. **2005** (in press).

(21) Rodriguez-Amaya, D. B. *A Guide to Carotenoids Analysis in Foods*; ILSI Press: Washington, DC, 1999.

(22) Kimura, M.; Rodriguez-Amaya, D. B. A scheme for obtaining standards and HPLC quantification of leafy vegetable carotenoids. *Food Chem*. **2002**, *78*, 389−398.

(23) Tee, E. S.; Lim, C. L. Carotenoid composition and content of Malaysian vegetables and fruits by the AOAC and HPLC methods. *Food Chem*. **1991**, *41*, 309−339.

(24) Bonnett, R.; Mallams, A. K.; McCormick, A.; Spark, A. A.; Tee, T. L.; Weedon, B. C. L. Carotenoids and related compounds. Part XX. Structure and reactions of fucoxanthin. *J. Chem. Soc. C* **1969**, 429−454.

(25) Britton, G., Liaaen-Jensen, S., Pfander, H., Eds. *Overview of carotenoid biosynthesis. In Carotenoids: Biosynthesis and Metabolism*; Birkhauser Verlag: Basel, Switzerland, 1998.

(26) Burns, J.; Fraser, P. D.; Bramley, P. M. Identification and quantification of carotenoids, tocopherol and chlorophylls in commonly consumed fruits and vegetables. *Phytochemistry* **2003**, *62*, 939−947.

(27) Emenhiser, C.; Sander, L.; Schwartz, S. Capability of a polymeric C_{30} stationary phase to resolve cis−trans carotenoid isomers in reversed-phase liquid chromatography. *J. Chromatogr. A* **1995**, *707*, 205.

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