Isolation and Structural Elucidation of (13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-Lutein from Marigold Flowers, Kale, and Human Plasma

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(13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-Lutein has been isolated and purified from extracts of marigold flowers, fresh raw kale (*Brassica oleracea* var. Acephala), and human plasma and fully characterized by ^{1}H and ^{13}C NMR, UV/vis, and MS. While the concentration of (13*Z*,13′*Z*)-lutein in kale and human plasma compared to (*all-E*,3*R*,3′*R*,6′*R*)-lutein was found to be quite low, this compound was readily isolated by fractional crystallization of lutein from marigold extracts. Thus, the mother liquors from two consecutive crystallizations of lutein from a saponified extract of marigold flowers were enriched in (13*Z*,13′*Z*)-lutein (8.7% of total carotenoids) and employed for the isolation of this compound by HPLC. The identity of the di-*Z*-lutein in kale and human plasma has been established by comparison of the HPLC−UV/vis−MS profiles of the purified compounds with those of a fully characterized sample, isolated from marigolds. 3-Hydroxy-*β*,*ε*-caroten-3′-one and 3′-epilutein have also been identified in extracts from marigolds.

Keywords: *Brassica oleracea (Acephala); carotenoid metabolites in plants; carotenoid oxidation products; HPLC−mass spectrometry; Z/E (cis/trans)-isomers; marigold flowers; new serum/plasma carotenoids; NMR; Tagetes erecta (orangeade); UV−visible*

INTRODUCTION

Carotenoids are one of the most abundant classes of phytochemicals found in nature, and the number of these compounds in the food supply, mainly in fruits and vegetables, has been estimated to be in excess of 40 (Khachik et al., 1991). Consumption of these fruits and vegetables results in absorption and metabolism of carotenoids by humans. The nutritional importance of carotenoids in prevention of cancer (Narisawa et al., 1996; Kim et al., 1997; King et al., 1997; Khachik et al., 1998) and macular degeneration (Seddon et al., 1994; Khachik et al., 1997a) has only recently been established from various interdisciplinary studies. As a result of these reports, the study of bioavailability and metabolism of dietary carotenoids in humans has taken center stage in recent years (Khachik et al., 1995a, 1997b; Paetau et al., 1998). However, without an extensive knowledge of the qualitative and quantitative distribution of carotenoids in human serum/plasma, these studies would not have been possible. To date, we have reported on isolation, separation, and characterization of 34 carotenoids including 9 of their metabolites in the extracts from human serum/plasma and milk (Khachik et al. 1992a,b, 1995b, 1997c). However, details of identification of several of these carotenoids have not yet been published.

Here, we wish to report on structural elucidation of (13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-lutein which, along with other common stereoisomers of dietary (*all-E*,3*R*,3′*R*,6′*R*)-lutein, has been previously detected in the human serum, milk, and retina (Khachik et al., 1997a,c). Although we have established that a low dietary concentration of this di-*Z*-lutein is present in a green vegetable such as fresh raw kale (*Brassica oleracea* var. Acephala), extracts from marigold flowers were found to serve as a more convenient source for the isolation and characterization of this compound.

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EXPERIMENTAL PROCEDURES

Apparatus. A Beckman model 114M binary solvent delivery system equipped with a Beckman model 421 controller was interfaced into a Hewlett-Packard (HP) 1040A rapid-scanning UV/visible photodiode array detector. The data were stored and processed by a HP 9000/Series 300 (Chem-Station) computing system, in combination with a HP model 9153B disk drive, model 35741 color display monitor, and a model 7470A plotter. The absorption spectra of the carotenoids were recorded between 200 and 600 nm at a rate of 12 spectra/min. This HPLC system was interfaced into a Hewlett-Packard model 5989A particle beam mass spectrometer. Eluate from the HPLC was divided with a ratio of 1:3 with the lesser amount entering the particle beam interface which was operated at a desolvation temperature of 45 °C. Electron capture negative ionization (ECNI) was achieved using methane at a pressure of 0.85 Torr and a source temperature of 250 °C. Spectra were collected from *m*/*z* 100 to 700 using a scan cycle time of 1.5 s.

Chromatographic Procedures. The analytical separations were carried out on both normal-phase (eluent A) and reversed-phase (eluents B and C) HPLC columns. Eluent A was also employed for semipreparative separations. Analytical separations were simultaneously monitored at 436 and 450 nm wavelengths, and preparative separation was monitored at 440 nm wavelength.

Normal-Phase Separations (Eluent A). These separations were carried out on a silica-based nitrile-bonded (25 cm

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length × 4.6 mm i.d.; 5 μm spherical particle) column (Regis Chemical Co., Morton Grove, IL), which was protected with a Brownlee nitrile-bonded guard cartridge (3 cm length × 4.6 mm i.d.; 5 μm particle size). For this separation an isocratic mixture of hexane (75%) and dichloromethane (25%), containing 0.25% methanol and 0.1% of *N*,*N*-diisopropylethylamine (DIPEA), at a column flow rate of 0.70 mL/min was employed. This eluent was also used as the HPLC injection solvent with the analytical and semipreparative normal-phase separations.

Reversed-Phase Separations (Eluents B and C). These separations were carried out on a Microsorb (25 cm length × 4.6 mm i.d.) C_{18} (5 μm spherical particles) column (Rainin Instrument Co., Wouburn, MA), which was protected with a Brownlee guard cartridge (3 cm length × 4.6 mm i.d.) packed with spheri-5-C_{18} (5 μm particle size).

Eluent B. A combination of isocratic and gradient HPLC employing a two-pump solvent module was used with this eluent. Pump A pumped a mixture of acetonitrile/methanol (9/1, v:v), and pump B pumped a mixture of hexane/dichloromethane/methanol/DIPEA (4.5/4.5/0.99/0.01, v:v:v:v). At time zero, an isocratic mixture of acetonitrile (85.5%), methanol (9.995%), dichloromethane (2.25%), hexane (2.25%), and DIPEA (0.005%) (95% pump A, 5% pump B) was pumped for 10 min. After 10 min, a linear gradient was run for 30 min resulting in a final composition of acetonitrile (40.5%), methanol (9.95%), dichloromethane (24.75%), hexane (24.75%), DIPEA (0.055%) (45% pump A, 55% pump B). The column flow rate was 0.70 mL/min. The HPLC injection solvent with this eluent consisted of a mixture of acetonitrile (40%), dichloromethane (20%), hexane (20%), and methanol (20%). At the end of the gradient, the column was equilibrated under the initial isocratic conditions for 15 min.

Eluent C. An isocratic mixture of acetonitrile (99%), methanol (1%), and DIPEA (0.1%) at a column flow rate of 1.2 mL/min was employed with this eluent. The HPLC injection solvent with this eluent consisted of a mixture of acetonitrile (80%), methanol (10%), dichloromethane (5%), and hexane (5%).

Semipreparative Normal-Phase Separations. These separations were carried out on a semipreparative silica-based nitrile-bonded (25 cm length × 10.0 mm i.d.; 5 μm spherical particle) column (Regis Chemical Co., Morton Grove, IL), which was protected with a Brownlee nitrile-bonded guard cartridge (3 cm length × 4.6 mm i.d.; 5 μm particle size). Eluent A at a flow rate of 3.0 mL/min was employed with this column.

UV−Visible Spectrophotometry and NMR Spectroscopy. Absorption spectra of the carotenoids in various solvents were recorded on a Beckman DU-7 UV−visible spectrophotometer.

All NMR experiments were performed on a Bruker DRX-400 spectrometer (^{1}H frequency: 400.13 MHz; ^{13}C frequency: 100.62 MHz); spectra were acquired at 23 °C in C_6D_6 (99.80% deuterium). Chemical shifts (δ) of protons and carbon-13 are given in units of ppm and are related to the residual solvent signals of benzene-d_6 (7.15 ppm in ^{1}H, 128.00 ppm in ^{13}C). The coupling constant (J) values are given in hertz. Other NMR experiments performed were H,H-COSY (homonuclear chemical shift correlated 2D spectroscopy), DEPT-135 (distortionless enhancement by polarization transfer) at 90° and 45° pulse angles, and inverse HMQC (hetero multiple quantum coherence spectroscopy). The application of these techniques for structural elucidation of carotenoids has been described in the literature (Englert et al., 1993; Englert, 1995). The multiplicity of the signals have been abbreviated as follows: s (singlet), d (doublet), and m (multiplet).

Reagents and Materials. Kale (*Brassica oleracea* var. Acephala*)* was purchased from a local supermarket. Saponified oleoresin of marigold flowers (*Tagate erecta* var. Orangeade*)* was donated by Kemin Industries (Des Moines, IA). Large quantities of human plasma were obtained from the American Red Cross (Baltimore, MD). Reference samples of β-carotene, β,ε-carotene-3-ol (α-cryptoxanthin), 3-hydroxy-β-carotene (β-cryptoxanthin), 3-hydroxy-β,ε-caroten-3′-one, and (*all-E*,3*R*,3′*S*,6′*R*)-β,ε-carotene-3,3′-diol [(*all-E*)-3′-epilutein] were from our collection of carotenoids (Khachik et al., 1992a). (*all-E*,3*R*,3′*R*,6′*R*)-β,ε-Carotene-3,3′-diol [(*all-E*,3*R*,3′*R*,6′*R*)-lutein], (*all-E*,3*R*,3′*R*)-β,β-carotene-3,3′-diol [(*all-E*,3*R*,3′*R*)-zeaxanthin], and their mono-*Z*-geometrical isomers were prepared according to published procedures (Khachik et al., 1992b). 3-Hydroxy-3′,4′-didehydro-β,γ-carotene (anhydrolutein) was obtained by partial synthesis (Khachik et al., 1995b). ε,ε-Carotene-3,3′-diol (lactucaxanthin) was isolated from a saponified extract of Romaine lettuce (*Lactuca sativa*) and further purified by preparative thin-layer chromatography followed by semipreparative normal-phase HPLC separation using eluent A. *N*,*N*-Diisopropylethylamine (DIPEA) was obtained from Aldrich Chemical Co. n-Silica gel (60−200 mesh) for flash column chromatography was purchased from J. T. Baker. HPLC solvents, acetonitrile, dichloromethane, hexane, and methanol (Fisher Scientific Products) were used without purification.

Extraction Procedures. Saponified oleoresin of marigold flowers was extracted and partially purified according to a published procedure to give approximately 70% pure lutein and a number of other minor carotenoids (Khachik, 1995).

Kale (Khachik et al., 1986, 1992c) and human plasma (Khachik et al., 1992a, 1997c) were extracted according to our earlier published procedures.

Isolation and Purification of (13*Z*,13′*Z*)-Lutein. *a. From Marigold Extracts.* Crystalline (3*R*,3′*R*,6′*R*)-lutein (5 g, 70% pure) obtained from a saponified extract of marigold flowers (Khachik, 1995) was dissolved in dichloromethane (200 mL) containing 0.1% DIPEA, and hexane (300 mL) was added. The mixture was kept in a freezer at −70 °C overnight to allow crystallization of lutein. Filtration gave lutein (3.5 g) which was shown by HPLC−UV/vis−MS and NMR (Khachik et al., 1992b) to be 98% pure. A small portion of the filtrate was examined by HPLC−UV/vis−MS using eluent A. The rest of the filtrate was evaporated to dryness and redissolved in dichloromethane (100 mL) and then treated with hexane (400 mL). The mixture was kept in a freezer at −70 °C overnight to allow recrystallization of lutein. After filtration, an additional amount of purified lutein (0.5 g) was collected, and the mother liquor from this second recrystallization was evaporated to dryness to give approximately 1 g of dark orange solids. The crude solid was redissolved in eluent A, microfiltered through 0.45 μm disposable filter assembly (Baxter, Scientific Product Division, McGaw Park, IL) and was then subjected to analytical and semipreparative normal-phase separation employing eluent A. In the order of HPLC elution, the carotenoids were separated by semipreparative HPLC (eluent A) and identified by comparison of their HPLC−UV/vis−MS profiles with those of standards (Table 1). These were as follows: β-carotene, a coeluting mixture of α-cryptoxanthin and β-cryptoxanthin [separated and identified by HPLC−UV/vis−MS using eluent B (Khachik et al., 1992a)], anhydrolutein, 3-hydroxy-β,ε-caroten-3′-one, ε,ε-carotene-3,3′-diol, an unknown which was later identified as (13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-lutein, (*all-E*,3*R*,3′*R*,6′*R*)-lutein, (*all-E*-,3*R*,3′*R*)-zeaxanthin, (*all-E*)-3′-epilutein, (9*Z*,3*R*,3′*R*,6′*R*)-lutein, (9′*Z*,3*R*,3′*R*,6′*R*)-lutein, (13*Z*,3*R*,3′*R*,6′*R*)-lutein, and (13′*Z*,3*R*,3′*R*,6′*R*)-lutein.

(13*Z*,13′*Z*-,3*R*,3′*R*,6′*R*)-Lutein (Figure 1) was fully characterized by comparison of its UV/visible absorption spectra in various solvents (Table 2) and its ^{1}H and ^{13}C NMR spectra with the data obtained for the *all-E*-compound. The observed ^{1}H and ^{13}C NMR chemical shift differences of the (13*Z*,13′*Z*)-lutein and *all-E*-lutein (Table 3) were consistent with the expected values reported by Englert (1995).

NMR ^{1}H: 1.45 (H−C-2ax, ψt, $J_{2ax}/J_{2eq} = J_{2ax/3} = 11.8$), 1.69 (H−C-2eq, ddd, $J_{2eq/3} = 3.7$, $J_{2eq/4eq} = 2.1$), 3.81 (H−C-3, dddd, $J_{3/2ax} = 11.8$, $J_{3/2eq} = 3.7$, $J_{3/4ax} = 9.2$, $J_{3/4eq} = 5.9$), 1.99 (H−C-4ax, dd, $J_{4ax/4eq} = 17.1$, $J_{4ax/3} = 9.2$), 2.24 (H−C-4eq, ddd, $J_{4eq/4ax} = 17.1$, $J_{4eq/3} = 5.9$, $J_{4eq/2eq} = 2.1$), 6.22 (H−C-7, d, $J_{7/8} = 15.9$), 6.35 (H−C-8, d, $J_{8/7} = 15.9$), 6.27 (H−C-10, d, $J_{10/11} = 11.2$), 6.73 (H−C-11, dd, $J_{11/10} = 11.2$, $J_{11/12} = 14.9$), 7.06 (H−C-12, d, $J_{12/11} = 14.9$), 6.14 (H−C-14, spin system of higher order), 6.86 (H−C-15, spin system of higher order), 1.089 (Me-16, s*), 1.100 (Me-17, s*), 1.759 (Me-18, s), 1.790 (Me-19, s), 1.923 (Me-20, s), 1.30 (H−C-2′a, dd, $J_{2'a/2'b} = 13.1$, $J_{2'a/3'} = 6.7$)**, 1.72 (H−C-2′b, dd, $J_{2'b/2'a} = 13.1$, $J_{2'b/3'} = 5.8$)**, 4.13

Table 1. HPLC Peak Identification of Carotenoids in Marigold Extracts from Their Wavelengths of Absorption Maxima and Mass Spectral Data Determined by HPLC Photodiode Array Detection Mass Spectrometry in the Order of Elution with Eluent A

peak no.	carotenoids[a,b]	absorption maxima (nm)[c]	molecular mass (*m*/z)[d]
1	β,β-carotene (β-carotene)	(430), 454, 478	536
2[e]	β,ε-carotene-3-ol (α-cryptoxanthin) + 3-hydroxy-β-carotene (β-cryptoxanthin)	(424), 448, 476	552
3[e]	3-hydroxy-3′,4′-didehydro-β,γ-carotene (anhydrolutein)	(424), 448, 476	550
4	3-hydroxy-β,ε-caroten-3′-one	(424), 448, 476	566, 548 (M−H_2O)
5[f]	ε,ε-carotene-3,3′-diol	416, 442, 470	568, 550 (M−H_2O)
6[f]	(13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol [(13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-lutein]	274, 336, 410, 432, 460	568, 550 (M−H_2O)
7[f]	(*all-E*, 3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol [(*all-E*, 3*R*,3′*R*,6′*R*)-lutein]	(424), 448, 476	568, 550 (M−H_2O)
8	(*all-E*,3*R*,3′*R*)-β,β-carotene-3,3′-diol [(*all-E*,3*R*,3′*R*)-zeaxanthin]	(428), 454, 482	568
9	(*all-E*,3*R*,3′S,6′*R*)-β,ε-carotene-3,3′-diol [(*all-E*)-3′-epilutein]	(424), 448, 476	568, 550 (M−H_2O)
10[f]	(9*Z*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol [(9*Z*,3*R*,3′*R*,6′*R*)-lutein]	334, (420), 442, 470	568, 550 (M−H_2O)
11[f]	(9′*Z*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol [(9′*Z*,3*R*,3′*R*,6′*R*)-lutein]	332, (420), 444, 472	568, 550 (M−H_2O)
12[f]	(13*Z*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol [(13*Z*,3*R*,3′*R*,6′*R*)-lutein]	270, 334, (416), 440, 468	568, 550 (M−H_2O)
13[f]	(13′*Z*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol [(13′*Z*,3*R*,3′*R*,6′*R*)-lutein]	272, 332, (418), 442, 468	568, 550 (M−H_2O)

[a] All carotenoids were also detected in human plasma. [b] Common names for certain carotenoids are shown in parentheses. [c] Values in parentheses represent points of inflection. [d] The molecular ions appeared as the base peak (100% intensity). [e] The coeluting HPLC peaks were further separated and identified by reversed-phase HPLC according to published procedures (Khachik et al., 1992a). [f] Carotenoids found in a saponified kale extract after the removal of β-carotene and carotenoid epoxides by flash column chromatography.

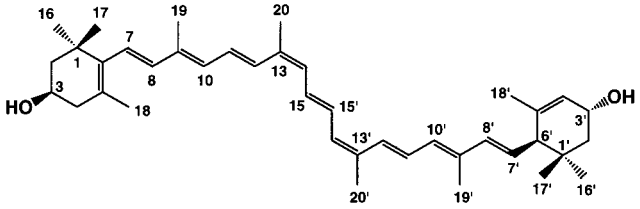

Figure 1. Stereochemistry of (13*Z*,13′*Z*, 3*R*,3′*R*,6′*R*)-lutein.

Table 2. UV/Visible Absorption Maxima of (13*Z*,13′*Z*)-Lutein and (*All-E*)-Lutein in Various Solvents

	absorption maxima[a] (nm)			
solvent	overtone	*Z*-peak	fine structure	[ϵ_2/ϵ_1][b]
hexane				
13*Z*,13′*Z*	272	332	408, (430.5), 457.5	0.130
all-E	*c*	330	422, (444), 473	0.127
ethanol				
13*Z*,13′*Z*	272	332	414 (inflection), (433.5), 459.5	0.152
all-E	*c*	333	424 (inflection), (445), 474	0.122
dichloromethane				
13*Z*,13′*Z*	277	341.5	418, (440), 467	0.137
all-E	*c*	337	433, (455), 484	0.047

[a] Values in parentheses represent main absorption maxima. [b] Ratio of absorption intensities (ϵ_2) at *Z*-peak in the near-UV region (330−342 nm) to absorption intensities (ϵ_1) at main absorption maxima. [c] An overtone absorption maximum was not observed.

(H−C-3′, m, not resolved), 5.53 (H−C-4′, m, not resolved), 2.31 (H−C-6′, d, $J_{6'/7'}$ = 9.7), 5.46 (H−C-7′, dd, $J_{7'/6'}$ = 9.7, $J_{7'/8'}$ = 15.6), 6.22 (H−C-8′, d, $J_{8'/7'}$ = 15.6), 6.34 (H−C-10′, d, $J_{10'/11'}$ = 11.0), 6.77 (H−C-11′, dd, $J_{11'/10'}$ = 11.0, $J_{11'/12'}$ = 14.8), 7.04 (H−C-12′, d, $J_{12'/11'}$ = 14.8), 6.14 (H−C-14′, spin system of higher order), 6.86 (H−C-15′, spin system of higher order), 1.007 (Me-16′, s), 0.824 (Me-17′, s), 1.624 (Me-18′, s), 1.890 (Me-19′, s), and 1.923 (Me-20′, s). *Assignment may be interchanged. **No assignment to axial or equatorial positions can be made.

Table 3. Comparison of the ^{1}H and ^{13}C Isomerization Shift Differences [$\Delta\delta = \delta(Z) - \delta(E)$] for (13*Z*,13′*Z*,3*R*,3′*R*,6′*R*)-Lutein with the Expected Literature (Englert, 1995) Values in $CDCl_3$

NMR signals	$\Delta\delta = \delta$(13*Z*,13′*Z*) − δ(*all-E*) ppm (literature)	$\Delta\delta = \delta$(13*Z*,13′*Z*) − δ(*all-E*) ppm (observed)
	^{1}H	
H−C-10	+0.04	+0.11
H−C-12	+0.52	+0.70
H−C-14	−0.15	−0.11
H−C-15	+0.09	+0.23
H−C-14′	−0.15	−0.11
H−C-12′	+0.52	+0.69
H−C-10′	+0.04	+0.20
	^{13}C	
C-12	−8.2	−7.5
C-14	−1.8	−1.0
C-15	−2.2	−1.4*
C-20	+7.8	+8.2**
C-20′	+7.8	+8.1**
C-15′	−2.2	−1.5*
C-14′	−1.8	−1.0
C-12′	−8.2	−7.5

*, **Interchangeable values.

NMR ^{13}C: 37.15 (C-1), 48.85 (C-2), 64.62 (C-3), 42.92 (C-4), 126.97 (C-5), 136.77 (C-6), 126.36 (C-7), 138.95 (C-8), 134.90 (C-9), 131.56 (C-10, overlapped with C-14 and C-14′ signals), 126.47 (C-11), 130.08 (C-12), 136.37 (C-13), 131.56 (C-14, overlapped with C-10 and C-14′ signals), 128.69 (C-15),* 28.85 (C-16),** 30.45 (C-17),** 21.76 (C-18), 12.79 (C-19), 20.89 (C-20),*** 34.05 (C-1′), 44.83 (C-2′), 65.56 (C-3′), 125.92 (C-4′), 137.89 (C-5′), 55.26 (C-6′), 129.49 (C-7′), 138.03 (C-8′), 134.80 (C-9′), 132.07 (C-10′), 126.55 (C-11′), 130.14 (C-12′), 135.76 (C-13′), 131.56 (C-14′, overlapped with C-10 and C-14 signals), 128.58 (C-15′),* 29.62 (C-16′), 24.56 (C-17′), 22.97 (C-18′), 13.13 (C-19′), and 20.81 (C-20′). *,**,***Assignments may be interchanged.

b. From Kale. An extract from fresh raw kale (1800 g) in 800 mL of tetrahydrofuran (THF) was treated with a 10% solution of methanolic KOH (800 mL), and the mixture was stirred for 4 h at room temperature under an atmosphere of

nitrogen. The mixture was concentrated on a rotary evaporator at 40 °C to a total volume of 600 mL. This was then partitioned between dichloromethane (500 mL) containing 0.1% DIPEA and 10% sodium chloride solution (700 mL). Methanol (100 mL) was added to break up the resulting emulsion, and the layers were allowed to separate. The dark yellow organic layer was removed and washed with 10% aqueous NaCl (4 × 500 mL) until all the water soluble chlorophyll derivatives were removed and the pH of the aqueous layer was neutral. The organic layer was dried over sodium sulfate and evaporated to dryness on a rotary evaporator, and the dark yellow residue (7.44 g) was redissolved in dichloromethane (30 mL) for purification by flash column chromatography.

A flash column (40 cm length × 4 cm i.d.) was packed with 90 g of n-silica gel (60−200 mesh) using petroleum ether (PE, bp 30−60 °C). This corresponded to the height of 32 cm for the packing. The above saponified extract was loaded onto the column using a total of 30 mL of dichloromethane/0.1% DIPEA, the column was sequentially eluted with PE and a combination of PE and acetone, and the following fractions were collected and combined according to thin-layer chromatography using PE/acetone:4/1.

fractions	eluent (volume, mL)
1 (colorless)	PE (300)
2−5 (dark orange)	PE/acetone:4/1 (each 125)
6−8 (dark yellow)	PE/acetone:4/1 (each 125)
9−16 (yellow)	PE/acetone:4/1 (each 125)

Fraction 1 was shown by TLC not to contain any carotenoid. The rest of the above combined fractions were examined by TLC (PE/acetone:4/1) as well as HPLC on C_{18} reversed-phase (eluent B) and normal-phase (eluent A) columns. The various carotenoids were tentatively identified by comparison of their HPLC−UV/vis−MS (eluents A and B) profiles with those of standards according to our published procedures (Khachik et al., 1986, 1992a,b, 1995b).

Fractions 2−5 (TLC, $R_f = 0.94$) were shown to be β-carotene (Table 1).

Fractions 6−8 (TLC, $R_f = 0.22$) were shown by HPLC−UV/vis−MS (eluent A) in the order of elution to consist of a mixture of ϵ,ϵ-carotene-3,3′-diol, (13*Z*,13′*Z*)-lutein, *all-E*-lutein, *all-E*-zeaxanthin, 9*Z*-lutein, 9′*Z*-lutein, 13*Z*-lutein, and 13′*Z*-lutein. Semipreparative separations (eluent A) gave pure (13*Z*,13′*Z*)-lutein which was identified by comparison of its HPLC−UV/vis−MS profile with the standard compound isolated earlier from Marigold extracts.

Fractions 9−16 (TLC, $R_f = 0.11$) were shown by HPLC−UV/vis−MS (eluent B) to be a mixture of carotenoid epoxides. These were tentatively identified according to our published procedures (Khachik et al., 1986) as neoxanthin, violaxanthin, lutein epoxide, and a number of their rearrangement products known as carotenoid 5,8-epoxides (Eugster, 1985).

c. From Human Plasma. An extract from a large volume of human plasma (800 mL) was chromatographed on n-silica gel plates (1000 μm thickness) using PE/acetone:70/30 (Khachik et al., 1992a). The band consisting of *all-E*-lutein and *all-E*-zeaxanthin as well as their stereoisomers was subjected to semipreparative HPLC (eluent A) employing the nitrile-bonded column. (13*Z*,13′*Z*)-Lutein was isolated and identified by comparison of its HPLC−UV/vis−MS (Table 1) profile with the fully characterized standard which was obtained from marigold extracts.

Allylic Alkylation of (13*Z*,13′*Z*)-Lutein. A small portion of a sample of (13*Z*,13′*Z*)-lutein (0.5 mg) purified by semipreparative HPLC (see above) was dissolved in dichloromethane (1 mL) and treated with 1 mL of a dilute solution of HCl in ethanol (0.5% V/V) at room temperature under an atmosphere of nitrogen. The course of the reaction was monitored by thin layer chromatography (TLC) on n-silica gel plates using hexane/acetone:4/1 as eluent. According to TLC, after 2 h all the (13*Z*,13′*Z*)-lutein ($R_f = 0.34$) had been converted to (13*Z*,13′*Z*)-3′-ethoxylutein ($R_f = 0.62$). The mixture was sequentially treated with DIPEA (0.2 mL) and 5% sodium bicarbonate (2 mL). The organic layer was removed, dried over sodium sulfate, filtered, and evaporated to dryness. The residue was redissolved in a mixture of acetonitrile 80%), methanol (10%), dichloromethane (5%), and hexane (5%), microfiltered through a 0.45 μm disposable filter assembly, and examined by HPLC−UV/vis−MS (eluent B). (13*Z*,13′*Z*)-3′-Ethoxylutein was identified from its UV/vis−MS profile [HPLC retention time = 18.61 min, λ_{max} = 436 nm, MS = 596 (molecular parent ion), 550 (loss of ethanol from the molecular parent ion)]. According to HPLC−UV/vis−MS, a small amount of *all-E*-3′-ethoxylutein [HPLC retention time = 20.91 min, λ_{max} = 446 nm, MS = 596 (molecular parent ion), 550 (loss of ethanol from the molecular parent ion)] was also formed.

RESULTS AND DISCUSSION

Although the mono-*Z*-isomers of carotenoids are quite common in natural products, di-*Z*-isomers of these compounds are less frequently encountered. The isolation and characterization of (13*Z*,13′*Z*, 3*R*,3′*R*,6′*R*)-lutein from the saponified extracts of marigold flowers and kale was first inspired by the presence of this compound in human serum, milk, and retina (Khachik et al., 1997a,c). On the basis of the UV−visible absorption (λ_{max} = 446 nm) and mass spectral data (m/z = 568) obtained from the HPLC−UV/vis−MS analysis (eluent A) of extracts from human serum, this compound was first tentatively identified as a di-*Z*-isomer of lutein. However, because of its low concentration, isolation of the sufficient quantity of this compound from human serum for structural elucidation by NMR presented a major problem. Therefore an attempt was made to identify an abundant source of this di-*Z*-lutein from natural products. For this purpose a saponified extract of marigold flowers was found to be most appropriate because lutein in large quantities could be readily isolated and purified from this plant (Khachik, 1995). Furthermore, in an earlier publication, we had isolated and tentatively identified a poly-*Z*-lutein from extracts of marigolds with the same HPLC−UV/vis−MS (eluent A) profile as that of the compound of interest found in human serum (Khachik, 1995). Therefore, in the course of purification of lutein from the crude extracts of marigolds, it became abundantly clear that the tentatively identified di-*Z*-lutein could be isolated by fractional recrystallization.

The first crystallization of a saponified extract of marigold flowers resulted in crystalline lutein which was found by HPLC−UV/vis−MS (eluent A) to be approximately 70% pure. While the concentration of the di-*Z*-lutein in this 70% pure lutein was found to be extremely low, the mother liquor from this crystallization was found to be an excellent source of this compound. The second recrystallization of lutein from the mother liquor obtained from the first crystallization provided even a better source of the di-*Z*-lutein. This is clearly shown in the HPLC profile in Figure 2. The relative distribution of carotenoids in the 70% pure lutein and the mother liquors from two consecutive crystallization were determined by HPLC (eluent A) and are shown in Table 4. (13*Z*,13′*Z*)-Lutein was therefore isolated by semipreparative HPLC (eluent A) from the mother liquor from the second recrystallization of lutein and was fully characterized from its ^{1}H and ^{13}C NMR, UV−visible, and mass spectral data. Although the circular dichroism (CD) spectrum of the isolated (13*Z*,-13′*Z*)-lutein was not measured, an absolute configuration of 3*R*,3′*R*,6′*R* was expected and assigned to this compound. This is because, based on ^{1}H NMR and CD

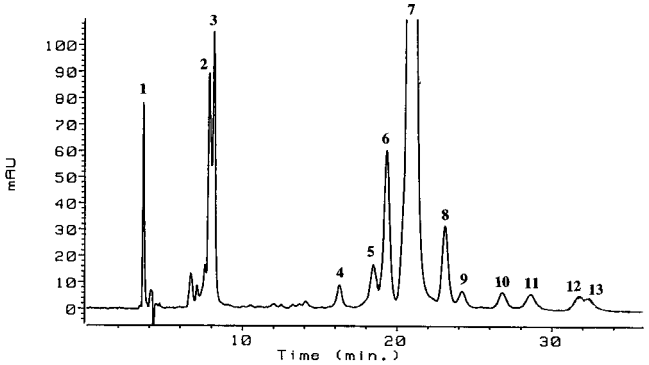

Figure 2. HPLC (eluent A) profile of carotenoids in the mother liquor from the second recrystallization of 70% pure lutein isolated from marigold flowers. For peak identification, see Table 1. HPLC conditions are described in text.

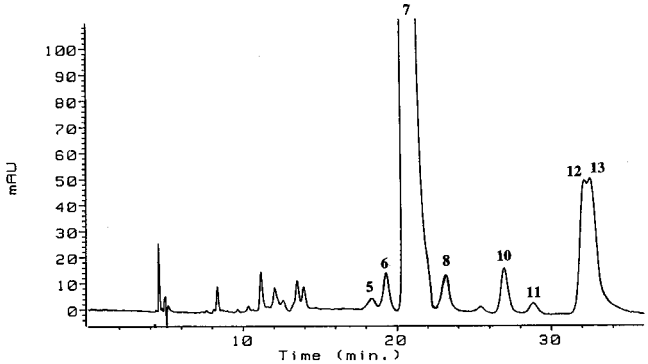

Figure 3. HPLC (eluent A) profile of carotenoids in the lutein fraction isolated by flash column chromatography of a saponified extract from kale. For peak identification, see Table 1. HPLC conditions are described in text.

Table 4. Relative Distribution of Carotenoids in 70% Pure Lutein (from Marigold Flowers) in Comparison with the Mother Liquors from Two Consecutive Recrystallizations of This Material

	relative distribution (%)[c]		
carotenoids[a,b]	70% pure lutein	mother liquor from first recrystallization	mother liquor from second recrystallization
β-carotene	0.4	1.3	2.9
α-cryptoxanthin + β-cryptoxanthin	0.05	1.9	4.7
anhydrolutein	0.06	2.1	4.9
3-hydroxy-β,ϵ-caroten-3′-one	0.1	0.3	0.7
ϵ,ϵ-carotene-3,3′-diol	0.3	0.8	1.0
(13Z,13′Z)-lutein	0.9	6.4	8.7
all-E-lutein	90.8	81.2	69.7
all-E-zeaxanthin	6.2	4.6	3.4
3′-epilutein	0.2	0.5	0.8
mono-Z-luteins[d]	0.03	0.9	3.2

[a] Carotenoids are listed in the order of elution on the nitrile-bonded column with eluent A. [b] Correct and systematic names are listed in Table 1. [c] The relative distribution of carotenoids were determined from the HPLC analysis of each mixture on a nitrile-bonded column (eluent A); conditions are described in text. [d] Z-Luteins were a mixture of 9Z-, 9′Z-, 13Z-, and 13′Z-lutein.

data, we have previously determined that the absolute configuration of *all-E*-lutein in extracts from human plasma and marigold flowers is 3*R*,3′*R*,6′*R*, which is identical to the dietary form of this compound found in fruits and vegetables (Khachik et al., 1992b; Khachik, 1995). Therefore the mono-*Z*- and the isolated di-*Z*-isomer of lutein would be expected to possess the same configuration as that of *all-E*-lutein. It is imperative to note that (13*Z*,13′*Z*)-lutein has also been isolated from the petals of rape *Brassica napus* and fully characterized by Kull and Pfander (1997).

In addition to lutein and its geometrical isomers, several other carotenoids common to marigolds were also separated by semipreparative HPLC and tentatively identified by comparison of their HPLC−UV/vis−MS profiles with those of carotenoid standards (Table 1). Among these, it is particularly interesting that two of the metabolites of lutein and zeaxanthin identified in human serum/plasma and retina, namely 3-hydroxy-β,ϵ-caroten-3′-one and 3′-epilutein, were also present in the extracts from marigolds (Khachik et al., 1992a, 1997a). The occurrence of these metabolites in plants is not unusual since both compounds have also been isolated from the flowers of *Caltha palustris* and fully characterized by Buchecker and Eugster (1979). It must be pointed out that in our experience with isolation of lutein from numerous plant sources, employing various solvents and chromatographic conditions, we have not observed isomerization of *all-E*-lutein to its *Z*-isomers (Khachik et al., 1986, 1992a,b, 1997a). Therefore, it is highly unlikely that the presence of (13*Z*,13′*Z*)-lutein in marigold flowers, kale, and human plasma reported here is an artifact of extraction, isolation, or chromatography.

The presence of (13*Z*,13′*Z*)-lutein in human plasma suggested that either this compound was formed in vivo as a result of isomerization of *all-E*-lutein, perhaps in the presence of acids in the human digestive system, or it may have originated from a dietary source. Because the most abundant source of dietary lutein among fruits and vegetables is found in kale (Khachik et al., 1986), an attempt was made to isolate the di-*Z*-lutein from this vegetable. After an extract of fresh raw kale was saponified to remove the chlorophylls and their derivatives, the carotenoid-containing residue was subjected to flash column chromatography. This allowed the separation of lutein and its *Z*-isomers from β-carotene and carotenoid epoxides, typically present in green plants (Khachik et al., 1986). The HPLC profile of the lutein fraction separated by flash column chromatography is shown in Figure 3. Although the concentration of (13*Z*,13′*Z*)-lutein was found to be very low in kale, a small quantity of this compound was separated from *all-E*-lutein by semipreparative HPLC. The identity of this compound was then established by comparison of its HPLC−UV/vis−MS profile with that of the fully characterized sample of (13*Z*,13′*Z*)-lutein isolated from marigold flowers. The saponified extract from kale also revealed the presence of a small amount of *all-E*-zeaxanthin. The ratio of *all-E*-lutein to *all-E*-zeaxanthin in kale, determined by HPLC (eluent A), was approximately 63/1.

In a similar approach, (13*Z*,13′*Z*)-lutein was also isolated and identified from an extract of human plasma; this confirmed our earlier report of the presence of this compound in the human serum (Khachik et al., 1997c).

Structural Elucidation of (13*Z*,13′*Z*)-Lutein. *a. NMR.* The assignment of all proton resonances were readily made by performing ^{1}H and COSY experiments. The differentiation between the spin systems H10/H11/H12 and H10′/H11′/H12′ was possible as cross-peaks between Me(19) ↔ H−C(10) and Me(19′) ↔ HC(10′) which were visible in the COSY spectrum. The protons of end groups of *all-E*-lutein and (13*Z*,13′*Z*, 3*R*,3′*R*,6′*R*)-lutein have similar chemical shifts, but these values are

not identical to data from literature (Englert, 1995). This is because our NMR data were obtained in benzene-d_6 as opposed to $CDCl_3$ reported by Englert (1995).

Complete ^{13}C-assignments could be obtained by analyzing the ^{13}C, DEPT-135, and inverse HMQC spectra. As in the case of protons, the end group resonances are similar but not identical to data published by Englert (1995). The resonances of quaternary carbons were identified by comparison with spectral data of carotenoids in benzene-d_6 obtained from our previous collection (Tarber, unpublished data, 1998).

The ^1H and ^{13}C NMR data of *all-E*-lutein in benzene-d_6 were not available, but those in $CDCl_3$ have been previously reported (Khachik et al., 1992b). In this case, the influence of solvent on ^1H and ^{13}C chemical shifts is significant but not extreme. This allowed comparison between the NMR data for the *all-E*-isomer of lutein obtained in $CDCl_3$ and those for the di-*Z*-isomer in benzene-d_6. This was based on the assumption that the characteristic isomerization shifts in the NMR spectra of *all-E*-lutein and its *Z*-isomers reported by Englert (1995) in $CDCl_3$ would also hold when the NMR of these carotenoids are obtained in benzene-d_6.

The ^1H and ^{13}C NMR isomerization shift differences for *all-E*-lutein and its (13*Z*,13′*Z*)-isomer are shown in Table 3. In (13*Z*,13′*Z*)-lutein both H−C(12), C(12), C(20) and H−C(12′), C(12′), C(20′) signals are drastically shifted, while signals due to H−C(14), H−C(15), C(14), C(15) as well as H−C(14′), H−C(15′), C(14′), C(15′) remain nearly unaffected. These results clearly indicate a 13*Z*,13′*Z* geometry for this lutein isomer. Therefore despite the use of benzene-d_6 rather than $CDCl_3$, the deviations between expected and found isomerization shift values are small.

Inspection of the olefinic signals in the proton NMR spectrum of the di-*Z*-lutein reported here reveals that this compound does not possess a 13*Z*,15*Z* or 13′*Z*,15′*Z* or 15*Z*,15′*Z* geometry. This is because in 13*Z*,15*Z*- or 13′*Z*,15′*Z*-isomers of lutein, the symmetry of the H14/H15/H15′/H14′ spin system is distorted, leading to a sort of ABMN spin system with unsymmetrical patterns for the signals due to H14/14′ and H15/15′ multiplets. In addition, the NMR data are not consistent with a (15*Z*,-15′*Z*)-lutein due to the absence of the $^3J_{HH}$ coupling (≈12 Hz) which would be expected between the *Z* (cis)-arranged H15 and H15′ (Englert, 1995). In contrast, in 13,13′-di-*Z*-lutein with a pseudo-AA′MM′ spin system, the NMR signals due H14/14′ and H15/15′ should appear as multiplets with nearly perfect symmetry and the $^3J_{HH}$ coupling constant value between the *all-E* (trans)-arranged H15 and H15′ would be expected to be around 15 Hz. Both latter cases are observed in the NMR data of the isolated di-*Z*-lutein, confirming a 13*Z*,13′*Z* geometry for this compound.

b. UV−Visible and MS. The UV−visible and MS data obtained by HPLC−UV/vis−MS for marigold carotenoids are shown in Table 1; these are in agreement with our previously published values for these compounds found in human serum and milk (Khachik et al., 1997c). The absorption spectrum of (13*Z*,13′*Z*)-lutein with a main maximum at 432 nm in the HPLC solvents (eluent A) shows a 16 nm hypsochromic shift from that of *all-E*-lutein (λ_{max} = 448 nm). As shown in Table 2, this hypsochromic shift in single solvents such as hexane, ethanol, and dichloromethane is in the range of 11−15 nm. These data are consistent with the presence of two *Z*-bonds located at or near the central carbons, i.e., 13, 13′, 15, or 15′, in the polyene chain of lutein. Since, based on the NMR data, the central C(15)−C(15′) double bond must exist in an *E* (trans)-geometry, the *Z*-bonds in the isolated di-*Z*-lutein should be located at the C(13) and C(13′) positions.

Similar to *all-E*-lutein and its mono-*Z*-isomers, the mass spectrum of (13*Z*,13′*Z*)-lutein, in addition to the molecular parent ion at m/z = 568, exhibits an ion at m/z = 550 due to the loss of water from the ϵ-end group of this compound (Table 1). Additional support for the presence of an allylic hydroxyl group in the isolated di-*Z*-lutein from marigold flowers was obtained from alkylation of this compound with ethanol in a dilute HCl solution. On the basis of their HPLC−UV/vis−MS profiles (eluent B), the products of this reaction were identified as (13*Z*,13′*Z*)-3′-ethoxylutein and *all-E*-3′-ethoxylutein. The formation of the latter compound was related to partial *Z*/*E*-isomerization of di-*Z*-lutein and/or its di-*Z*-ethoxylated products. The mass spectra of both ethoxylated compounds showed ions at m/z = 550 due to the loss of ethanol from their respective molecular parent ions.

SUMMARY AND CONCLUSION

In this report we have presented evidence that clearly confirms our earlier identification of (13*Z*,13′*Z*)-lutein in human serum and milk (Khachik et al., 1997c). We have shown that low levels of this di-*Z*-lutein can be found in a green vegetable such as kale. Although not determined at present, this suggests that other lutein-rich fruits and vegetables may also provide a dietary source of this compound. Even if this is the case, it is not known whether the presence of (13*Z*,13′*Z*)-lutein in human serum or milk is exclusively due to its selective uptake and absorption from the diet. Alternatively, (13*Z*,13′*Z*)-lutein may be the product of in vivo isomerization of *all-E*-lutein in the presence of acids in the humans digestive system. Nevertheless, while the *Z*-isomers of carotenoids always accompany their *all-E* counterparts in human serum and tissues, the biological function of these compounds remains unexplored.

NOMENCLATURE

For convenience, the trivial names of certain carotenoids have been used throughout this text. The trivial and correct systematic names for these carotenoids have been presented in Table 1. The terms *all-E*- and *Z*-isomers of carotenoids refer to all-trans and cis isomers of carotenoids, respectively. For in-chain geometrical isomers of carotenoids, the terms all-trans and cis, which have been used with the old nomenclature, are no longer appropriate.

LITERATURE CITED

Buchecker, R.; Eugster, C. H. Search for the presence in egg yolk, in flowers of *Caltha palustris* and in autumn leaves of 3′-epilutein and 3′,O-didehydrolutein. *Helv. Chim. Acta* **1979**, *62*, 2817−2824.

Englert, G.; Aakermann, T.; Liaaen-Jensen, S. NMR studies on natural all-trans-(9′Z,11′Z)−(3R,3′S,5′R,6′R)-pyrrhoxanthin, an acetylenic-C-37-skeletal nor-carotenoid butenolide. *Magn. Reson. Chem.* **1993**, *31*, 910−915.

Englert, G. NMR of carotenoids. In *Carotenoids*; Britton, G., Liaaen-Jensen, S., Pfander, H., Eds.; Birkhäuser: Basel, 1995; Vol. 1B, pp 147−260.

Eugster, C. H. Carotenoid structures, old and new problems. *Pure Appl. Chem.* **1985**, *57*, 639−647.

Khachik, F.; Beecher, G. R.; Whittaker, N. F. Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in the extracts of several green vegetables by liquid chromatography. *J. Agric. Food Chem.* **1986**, *34*, (4), 603−616.

Khachik, F.; Beecher, G. R.; Goli, M. B.; Lusby, W. R. Separation, identification, and quantification of carotenoids in fruits, vegetables and human plasma by high performance liquid chromatography. *Pure Appl. Chem.* **1991**, *63* (1), 71−80.

Khachik, F.; Beecher, G. R.; Goli, M. B.; Lusby, W. R.; Smith, J. C. Separation and identification of carotenoids and their oxidation products in extracts of human plasma. *Anal. Chem.* **1992a**, *64* (18), 2111−2122.

Khachik, F.; Englert, G.; Daitch, C. E.; Beecher, G. R.; Lusby, W. R.; Tonucci, L. H. Isolation and structural elucidation of the geometrical isomers of lutein and zeaxanthin in extracts from human plasma *J. Chromatogr. Biomed. Appl.* **1992b**, *582*, 153−166.

Khachik, F.; Goli, M. B.; Beecher, G. R.; Holden, J.; Lusby, W. R.; Tenorio, M. D.; Barrera, M. R. The effect of food preparation on qualitative and quantitative distribution of major carotenoid constituents of tomatoes and several green vegetables. *J. Agric. Food Chem.* **1992c**, *40* (3), 390−398.

Khachik, F. Process for Isolation, Purification, and Recrystallization of Lutein from Saponified Marigolds Oleoresin and Uses Thereof. U.S. Patent 5,382,714, Jan 17, 1995.

Khachik, F.; Beecher, G. R.; Smith, J. C., Jr. Lutein, lycopene, and their oxidative metabolites in chemoprevention of cancer. *J. Cell. Biochem.* **1995a**, *22*, 236−246.

Khachik, F.; Englert, G.; Beecher, G. R.; Smith, J. C., Jr. Isolation, structural elucidation, and partial synthesis of lutein dehydration products in extracts from human plasma. *J. Chromatogr. Biomed. Appl.* **1995b**, *670*, 219−233.

Khachik, F.; Bernstein, P.; Garland, D. L. Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. *Invest. Ophthalmol. Vis. Sci.* **1997a**, *38*, 1802−11.

Khachik, F.; Steck, A.; Pfander, H. Bioavailability, metabolism, and possible mechanism of chemoprevention by lutein and lycopene in humans. In *Food Factors for Cancer Prevention*; Ohigashi, H., Osawa, T., Terao, J., Watanabe, S., Yoshikawa, T., Eds.; Springer-Verlag: Tokyo, 1997b; pp 542−547.

Khachik, F.; Spangler, C. J.; Smith J. C., Jr.; Canfield, L. M.; Pfander, H.; Steck, A. Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. *Anal. Chem.* **1997c**, *69*, 1873−1881.

Khachik, F.; Bertram, J. S.; Huang, M. T.; Fahey, J. W.; Talalay, P. Dietary carotenids and their metabolites as potentially useful chemopreventive agents against cancer. Proceedings of the International Symposium on Antioxidant Food Supplements in Human Health, Kaminoyama-City, Yamagata Prefecture, Japan, Oct 16−18, 1997; Packer, L., Hiramatsu, M., Yoshikawa, T., Eds.; Academic Press: Tokyo, in press.

Kim, D. J.; Takasuka, N.; Kim, J. M.; Kazunori, S.; Ota, T.; Asamoto, M.; Murakoshi, M.; Nishino, H.; Nir, Z.; Tsuda, H. Chemoprevention by lycopene of mouse lung neoplasia after combined initiation treatment with DEN, MNU, and DMH. *Cancer Lett.* **1997**, *120*, 15−22.

King, T. J.; Khachik, F.; Bortkiewicz, H.; Fukishima, L. H.; Morioka, S.; Bertram, J. S. Metabolites of dietary carotenoids as potential cancer preventive agents. *J. Pure Appl. Chem.* **1997**, *69*, 2135−40.

Kull, D. R.; Pfander, H. Isolation and structural elucidation of two (Z)-isomers of lutein from the petals of rape Brassica napus. *J. Agric. Food Chem.* **1997**, *45*, 4201−4203.

Narisawa, T.; Fukaura, Y.; Hasebe, M.; Ito, M.; Aizawa, R.; Murakoshi, M.; Uemura, S.; Khachik, F.; Nishino, H. Inhibitory effects of natural carotenoids, α-carotene, *β*-carotene, lycopene and lutein on colonic aberrant crypt foci formation in rats. *Cancer Lett.* **1996**, *107*, 137−142.

Paetau, I.; Khachik, F.; Brown, E. D.; Beecher, G. R.; Kramer, T. R.; Chittams, J.; Clevidence, B. A. Chronic ingestion of lycopene-rich tomato juice or lycopene supplements significantly increases plasma concentrations of lycopene and related tomato carotenoids in humans. *Am. J. Clin. Nutr.* **1998**, *68*, 1187−1195.

Seddon, J. M.; Ajani, U. A., Sperduto, R. D.; Hiller, R.; Blair, N.; Burton, T. C.; Farber, M. D.; Gragoudas, E. S.; Haller, J.; Miller, D. T.; Yannuzzi, L. A.; Willett, W. Dietary carotenoids, vitamin A, C, and E, and advanced age-related macular degeneration. *J. Am. Med. Assoc.* **1994**, *272*, 1413−1420.

Tarber, B. Unpublished Results, University of Bern, June 1998.

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