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Isolation and structural elucidation of the geometrical isomers of lutein and zeaxanthin in extracts from human plasma

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ABSTRACT

All-*E*-(3*R*,3′*R*,6′*R*)-lutein, all-*E*-(3*R*,3′*R*)-zeaxanthin, all-*E*-(3*R*,3′*S*,6′*R*)-3′-epilutein and some geometrical isomers of the former two dihydroxycarotenoids have been separated from an extract of human plasma by semipreparative high-performance liquid chromatography on a silica-based nitrile-bonded column. In the order of chromatographic elution, the isolated fractions were identified as all-*E*-lutein, all-*E*-zeaxanthin, all-*E*-3′-epilutein, 9*Z*-lutein, 9′*Z*-lutein, a mixture of 13*Z*-lutein and 13′*Z*-lutein, 9*Z*-zeaxanthin, 13*Z*-zeaxanthin and 15*Z*-zeaxanthin. The structures of all compounds, including the relative configuration at C(3′) and C(6′) of the luteins and the position of the stereomutated double bonds in the geometrical isomers, were unambiguously established by ^{1}H nuclear magnetic resonance spectroscopy. The absolute configuration of the three all-*E* compounds was derived by circular dichroism and is also assumed to be valid for the geometrical isomers. The ultraviolet–visible absorption and mass spectra of each of the individually isolated compounds were also in agreement with the proposed structures.

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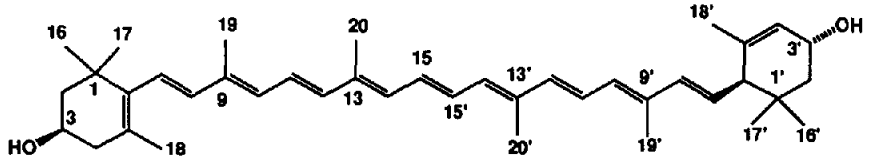
INTRODUCTION

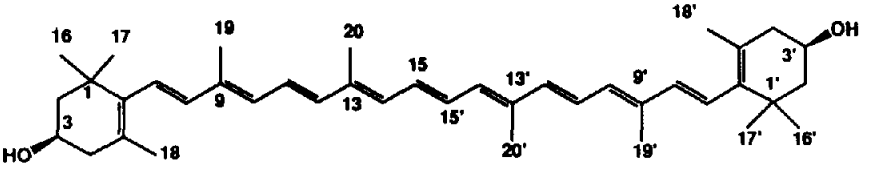
In the past decade, the analysis of carotenoids in foods and human plasma has become increasingly important owing to the association of the carotenoid-containing foods with prevention of several types of human cancers [1]. High-performance liquid chromatography (HPLC) has been widely employed as the method of choice for the separation of the various classes of carotenoids in extracts from foods and human plasma [1–9]. The separation of carotenoids containing various end-groups is normally accomplished on C_{18} reversed-phase HPLC columns with great success. However, certain polar oxygenated carotenoids and their stereoisomers are not effectively separated on reversed-phase HPLC adsorbents. Among these are lutein (**1**, see Fig. 1) and zeaxanthin (**2**), which are two of the most abundant dihydroxycarotenoids in most fruits and vegetables and human plasma. Although partial separation of **1** and **2** by HPLC can be accomplished on a C_{18} reversed-phase column [5], normal-phase adsorbents are found to be more suitable. More recently, the synthesis, isolation and full spectroscopic characterization of eleven *Z*-isomers of **1** which were separated on a Spherisorb S5W, Silica B 13/255 column have been described [10].

However, in HPLC separation of carotenoids from human plasma, we have recently shown



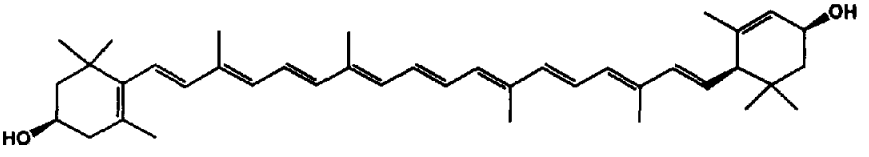
(3R,3'R,6'R)-β,ε-Carotene-3,3'-diol (Lutein)

1 (all-E)
4 (9Z)
5 (9'Z)
6 (13Z)
7 (13'Z)
8 (15Z)



(3R,3'R)-β,β-Carotene-3,3'-diol (Zeaxanthin)

2 (all-E)
9 (9Z)
10 (13Z)
11 (15Z)



(3R,3'S,6'R)-β,ε-Carotene-3,3'-diol (3'-Epilutein)

3 (all-E)

Fig. 1. Chemical structures of lutein, zeaxanthin and 3'-epilutein.

that a nitrile-bonded column results in excellent separation of a mixture of keto- and hydroxycarotenoids including **1, 2** and **3** (Fig. 1) [1,11]. In addition to the all-*E*-isomers, several geometrical isomers of lutein (9*Z*, 9′*Z*, mixture of 13*Z* and 13′*Z*) and zeaxanthin (9*Z*, 13*Z*, 15*Z*) were fully resolved and separated. However, the details of the separation and structural elucidation of the geometrical isomers of lutein and zeaxanthin were not described in our previous publications [1,11].

In this report we present the detailed separation and structural elucidation of the most prominent geometrical isomers of lutein and zeaxanthin in extracts from human plasma. These geometrical isomers have been separated by semipreparative HPLC on a nitrile-bonded column and have been identified by ^{1}H nuclear magnetic resonance (NMR) (400 MHz) spectroscopy. Additional evidence for the structural assignments has been obtained from the UV–visible absorption, circular dichroism (CD) and mass spectra of the isolated and purified carotenoids.

EXPERIMENTAL

Apparatus

A Beckman Model 114M single solvent delivery system equipped with a Beckman Model 421 controller was interfaced into a Waters 990 rapid-scanning UV visible photodiode-array detector. The data were stored and processed by means of a NEC APC IV computing system, which was operated with a color display monitor, Model APC-H431, and a Waters 990 plotter. The absorption spectra of the carotenoids were recorded between 200 and 600 nm at a rate of twelve spectra per min; spectra for examination and publication were selected from the apex of each peak in an attempt to minimize contamination in those cases where peaks were not completely resolved. Absorption spectra of the carotenoids in various solvents were recorded on a Beckman DU-7 UV visible spectrophotometer. The ^{1}H NMR spectra were obtained on a Bruker-WM-400 and AM-400-FT (400 MHz) in $CDCl_3$ at *ca.* 26°C with trimethylsilane (TMS) as internal standard.

The CD spectra were obtained on a Jasco J-720 spectropolarimeter which was equipped with an IBM compatible PC Model 386 data processor. The CD spectra were collected in EPA (diethyl ether–isopentane–ethanol, 5:5:2) solution between 220 and 400 nm with a sensitivity of 50 mdeg/cm and a scan speed of 50 nm/min. Mass spectra were obtained from a Finnigan-MAT Model 4510 mass spectrometer (San Jose, CA, USA) equipped with an INCOS data system and a direct exposure probe which was heated by the application of current from 0 to 1000 mA at a rate of 50 mA/s. Negative-ion electron-capture (ECNI) mass spectra were produced by using methane as a buffer gas at an indicated pressure of 30.39 kPa (0.3 Torr). For negative-ion spectra, the ionizing chamber was maintained at 60°C and spectra were collected from *m/z* 45 to *m/z* 650.

Chromatographic procedures

The semipreparative separations were carried out employing eluent A. This eluent consisted of an isocratic mixture of hexane (74.45%), dichloromethane (25%), methanol (0.45%) and N,N-diisopropylethylamine (0.10%). The separations with this eluent were carried out on a Regis (Morton Grove, IL, USA) stainless-steel (25 cm × 10 mm I.D.) nitrile-bonded column (5-μm particles), which was protected with a Brownlee nitrile-bonded guard cartridge (3 cm × 4.6 mm I.D.; 5-μm particles). The flow-rate with this eluent was 2.5 ml/min. The monitoring wavelength was 445 nm.

Reagents and materials

All-*E*-lutein (**1**) was isolated from kale (*Brassica oleracea*, Var. acephala) according to a published procedure [2]. All-*E*-zeaxanthin (**2**) was synthesized according to published procedures [12,13]. All-*E*-3′-epilutein (**3**) was prepared from lutein as published by Buchecker *et al.* [14].

Isolation of the geometrical isomers of lutein and zeaxanthin from human plasma

A large volume (880 ml) of human plasma (Amercian Red Cross) was extracted and chromatographed on C_{18} reversed-phase plates ac-

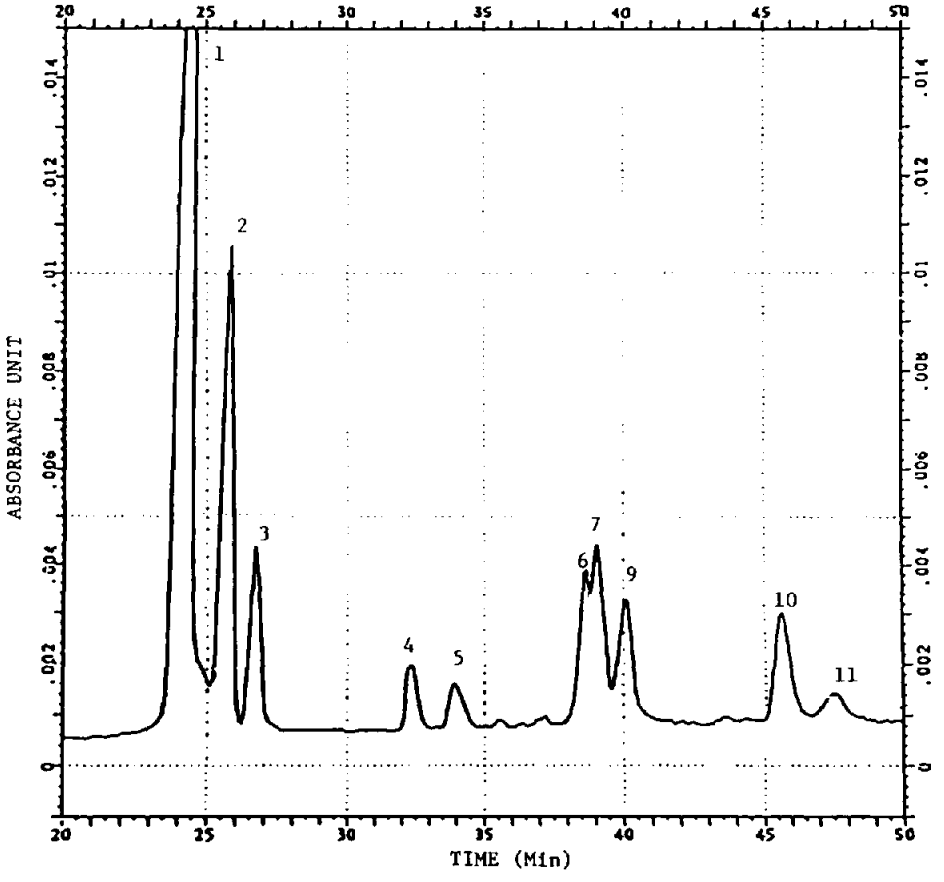


Fig. 2. HPLC separation of the stereoisomers of lutein and zeaxanthin in an extract from human plasma on a nitrile-bonded column. HPLC (eluent A) conditions described in text. For peak identification see Fig. 1.

cording to a published procedure [11]. The band consisting of all-*E*-lutein and all-*E*-zeaxanthin as well as their stereoisomers was subjected to semi-preparative HPLC on a nitrile-bonded column employing eluent A (Fig. 2). In the order of chromatographic elution, the following isomers of lutein and zeaxanthin where shown to be present.

All-E-(3R,3'R,6'R)-lutein (1). UV–visible absorption: see Tables I and II. Mass spectrum (ECNI, methane): molecular anion peak at *m/z* 568 (100%) and an anion peak at *m/z* 550 (20%, $[M - H_2O]^-$). CD in EPA solution, (λ, mdeg): 245 (+9.1), 275 (0) and 285 (−4.2) (in agreement with published values [15,16]). ^{1}H NMR (Table III) in agreement with literature values [15,17].

All-E-(3R,3'R)-zeaxanthin (2). UV–visible absorption: see Tables I and II. Mass spectrum (ECNI, methane): molecular anion peak at *m/z* 568 (100%) as well as anion peaks at *m/z* 550 (55%, $[M - H_2O]^-$) and *m/z* 532 (15%, $[M - 2H_2O]^-$). Mass spectrum [ECNI, deuteroammonia (ND_3)]: molecular anion peak at *m/z* 570 (70%) indicated two exchangeable hydrogens. CD in EPA solution (λ, mdeg): 223 (−22.51), 246 (+20.77), 284 (−31.93), 342 (+7.625) (in agreement with published values [10]). ^{1}H NMR (400 MHz): 1.074 [12H, H_3C(16,16',17,17')]; 1.36 [2H, OH-C(3) and OH-C(3')]; 1.48 [2H, H_{ax}-C(2) and H_{ax}-C(2')]; 1.738 [6H, H_3C(18,18')]; 1.77 [2H, H_{eq}-C(2) and H_{eq}-C(2')]; 1.974 [12H, H_3C (19,19',20,20')]; 2.04 [2H, H_{ax}-C(4) and H_{ax}-C4')]; 2.39 [2H, H_{eq}-C(4) and H_{eq}-C(4')]; 4.00 [2H, H_{ax}-C(3) and H_{ax}-C(3')]; 6.12 [2H, H-C(7) and H-C (7')]; 6.14 [2H, H-C(8) and H-C(8')]; 6.17 [2H,

TABLE I

UV VISIBLE ABSORPTION MAXIMA OF THE GEOMETRICAL ISOMERS OF LUTEIN AND ZEAXANTHIN ISOLATED AND PURIFIED BY SEMIPREPARATIVE HPLC IN VARIOUS SOLVENTS

Carotenoids	Absorption maxima[a,b] (nm), $[\varepsilon_2/\varepsilon_1]$[c]			
	Hexane	Ethanol	Acetone	Dichloromethane
Lutein				
all-*E*	330,(444),473,[0.127]	333,(445),474,[0.122]	336,(448),475,[0.119]	337,(455),484,[0.047]
9*Z*	330,(439),468,[0.117]	332,(440),469,[0.119]	336,(442),470,[0.140]	336,(449),478,[0.123]
9′*Z*	329,(440),468,[0.124]	331,(441),469,[0.140]	335,(443),470,[0.162]	337,(450),478,[0.116]
13*Z* + 13′*Z*[d]	330,(438),466,[0.443]	332,(439),467,[0.455]	336,(441),469,[0.336]	337,(447),475,[0.398]
15*Z*[e]	330,(441),469,[0.549]	332,(442),473,[0.569]	336,(444),473,[0.480]	337,(451),479,[0.494]
Zeaxanthin				
all-*E*	338,(451),477,[0.085]	340,(452),478,[0.076]	344,(454),481,[0.075]	347,(461),489,[0.066]
9*Z*	342,(445),473,[0.175]	344,(448),473,[0.236]	348,(449),474,[0.266]	351,(456),485,[0.222]
13*Z*	337,(443),470,[0.455]	339,(446),472,[0.465]	341,(447),473,[0.475]	345,(454),481,[0.462]
15*Z*	336,(447),474,[0.579]	337,(450),475,[0.634]	339,(451),477,[0.621]	345,(458),486,[0.629]

[a] Values in parentheses represent main absorption maxima.

[b] Points of inflection in the absorption spectra of carotenoids are not reported.

[c] Ratio of absorption intensities (ε_2) at *Z*-peak in the near-UV region (328–347 nm) to absorption intensities (ε_1) at main absorption maxima.

[d] Mixture of two isomers which could not be separated by HPLC under conditions employed.

[e] 15*Z*-Lutein was not isolated from human plasma, but was formed by I_2-induced isomerization of all-*E*-lutein.

TABLE II

UV–VISIBLE ABSORPTION MAXIMA OF THE GEOMETRICAL ISOMERS OF LUTEIN AND ZEAXANTHIN DETERMINED BY A PHOTODIODE-ARRAY DETECTOR IN THE HPLC SOLVENTS (ELUENT A)

	Absorption maxima[a,b] (nm)			
	Overtone	*Z*-peak	Fine structure	$[\varepsilon_2/\varepsilon_1]$[c]
Lutein				
all-*E*	270	334	(448) 476	[0.130]
9*Z*	270	334	(442) 470	[0.118]
9′*Z*	268	332	(444) 472	[0.110]
13*Z*	270	334	(440) 468	[0.489]
13′*Z*	272	332	(442) 468	[0.421]
15*Z*	270	334	(446) 474	[0.538]
Zeaxanthin				
all-*E*	274	338	(454) 482	[0.067]
9*Z*	274	340	(450) 474	[0.119]
13*Z*	274	338	(446) 472	[0.509]
15*Z*	272	338	(450) 478	[0.638]

[a] Values in parentheses represent main absorption maxima.

[b] Points of inflection in the absorption spectra of carotenoids are not reported.

[c] Ratio of absorption intensities (ε_2) at *Z*-peak in the near-UV region (332–340 nm) to absorption intensities (ε_1) at main absorption maxima.

TABLE III

^{1}H NMR DATA FOR ALL-*E*- AND FOUR MONO-*Z*-ISOMERS OF LUTEIN

δ in ppm; shift differences $\Delta\delta = \delta(Z) - \delta(E)$ for tabulated values > 0.02 ppm; at 400 MHz in $CDCl_3$.

	1 (all-*E*)	4 (9*Z*)		5 (9′*Z*)		6 (13*Z*)[a]		7 (13′*Z*)[a]	
	δ	δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$
H_{ax}-C(2)	1.48	1.50		1.48		1.49		1.48	
H_{ax}-C(2′)	1.37	1.37		1.38		1.37		1.37	
H_{eq}-C(2)	1.78	1.79		1.78		1.78		1.78	
H_{eq}-C(2′)	1.84	1.85		1.86		1.85		1.85	
H_{ax}-C(3)	4.00	4.03		4.00		4.00		4.00	
H_{eq}-C(3′)	4.25	4.25		4.27		4.25		4.25	
H_{ax}-C(4)	2.05	2.09		2.05		~2.05		~2.05	
H_{eq}-C(4)	2.39	2.43	0.04	2.39		N.O.[b]		N.O.[b]	
H-C(4′)	5.55	5.55		5.56		5.55		5.55	
H-C(6′)	2.41	2.41		2.48	0.07	N.O.		N.O.	
H-C(7)	6.12	6.13		6.10		6.10		6.10	
H-C(7′)	5.43	5.43		5.46	0.03	5.43		5.46	0.03
H-C(8)	6.12	6.67	0.55	6.12		6.10		6.10	
H-C(8′)	6.15	6.14		6.66	0.51	6.14		6.14	
H-C(10)	6.16	6.07	−0.09	6.16		6.21	0.06	6.14	
H-C(10′)	6.14	6.13		6.03	0.12	6.15		6.21	0.06
H-C(11)	6.64	6.74	0.10	6.64					
						6.65		6.64	
H-C(11′)	6.62	6.61		6.75	0.13				
H-C(12)		6.30	−0.06	6.36		6.90	0.54	6.37	
	~6.36								
H-C(12′)		6.35		6.30	−0.06	6.36		6.89	0.53
H-C(14)						6.14	−0.12	6.24	
	~6.26	~6.25		~6.25					
H-C(14′)						6.24		6.14	−0.12
H-C(15)						6.80	0.17	6.56	−0.07
	~6.63	~6.63		~6.64					
H-C(15′)						6.56	−0.07	6.80	0.17
CH_3(16)									
	1.073	1.084[c]		1.074		1.082		1.076	
		1.093[c]							
CH_3(17)									
CH_3(16′)									
	0.848[c]	0.849[c]		0.861[c]		0.850[c]		0.855[c]	
	0.998[c]	0.999[c]		1.025[c]		1.000[c]		1.007[c]	
CH_3(17′)									
CH_3(18)	1.737	1.775	0.04	1.737		1.745		1.739	
CH_3(18′)	1.625	1.625		1.646		1.632		1.632	
CH_3(19)	1.968	1.963		1.973[c]		1.956[c]		1.967[c]	
CH_3(19′)	1.910	1.909		1.910		1.908		1.917	
CH_3(20)				1.973[c]		1.975[c]		1.967[c]	
	1.968	1.963							
CH_3(20′)				1.986[c]		1.995[c]		1.986[c]	

[a] NMR data for an isomeric mixture of 13*Z* (~45%) and 13′*Z*-lutein (~55%). Chemical shifts were assigned by comparison of the NMR spectra of the mixture with those of pure standards.

[b] N.O. = not observed.

[c] These assignments may be interchanged.

H-C(10) and H-C(10′)]; 6.26 [2H, H-C(14) and H-C(14′)]; 6.36 (2H, H-C(12) and H-C(12′)]; 6.63 [2H, H-C(15) and H-C(15′)]; 6.64 [2H, H-C(11) and H-C(11′)]; These chemical shift values were in agreement with previously published values [10].

*All-E-3′-epilutein (**3**).* UV–visible (nm) absorption: hexane, λ_{max} = 444, 473 (lit. λ_{max} = 445, 472 [18]). Mass spectrum (ECNI, methane): molecular anion peak at *m/z* 568 (20%) as well as anion peaks at *m/z* 550 (100%, $[M - H_2O]^-$) and *m/z* 532 (60%, $[M - 2H_2O]^-$). CD in EPA solution (λ, mdeg): 242 (+4.44), 269 (0), 280 (−1.74), 296 (0), 334 (+1.97) (in agreement with published values [18]). ^{1}H NMR (400 MHz): 0.852 and 0.943 [6H, H_3C(16′,17′)]; 1.073 [6H, H_3C(16,17)]; 1.644 [3H, H_3C(18′)]; 1.737 [3H, H_3C(18)]; 1.907 [3H, H_3C(19′)]; 1.967 [9H, H_3C (19,20,20′)]; 2.03 [1H, H_{ax}-C(4)]; ~2.16 (J = 9.7 Hz) [1H, H-C(6′)]; 2.39 [1H, H_{eq}-C(4)]; *ca.* 4.0 [1H, H_{ax}-C(3)]; *ca.* 4.23 (1H, H_{ax}-C(3′)]; 5.485 [1H, H-C(4′)]; 5.532 [1H, H-C(7′)]; 6.1–6.7 (m, vinylic-H). These chemical shift values were in agreement with published values [18].

*9Z-Lutein (**4**).* UV–visible absorption: see Tables I and II. Mass spectrum (ECNI, methane): molecular anion peak at *m/z* 568 (50%) as well as anion peaks at *m/z* 550 (100%, $[M - H_2O]^-$) and *m/z* 532 (5%, $[M - 2H_2O]^-$). ^{1}H NMR see Table III.

*9′Z-Lutein (**5**).* UV–visible absorption: see Tables I and II. Mass spectrum (ECNI, methane): molecular anion peak at *m/z* 568 (48%) as well as anion peaks at *m/z* 550 (100%, $[M - H_2O]^-$) and *m/z* 532 (5%, $[M - 2H_2O]^-$). ^{1}H NMR see Table III.

*13Z-Lutein (**6**) plus 13′Z-lutein (**7**).* UV–visible absorption: see Tables I and II. Mass spectrum (ECNI, methane): molecular anion peak at *m/z* 568 (30%) as well as anion peaks at *m/z* 550 (100%, $[M - H_2O]^-$) and *m/z* 532 (20%, $[M - 2H_2O]^-$). ^{1}H NMR see Table III.

*9Z-Zeaxanthin (**9**).* UV–visible absorption: see Tables I and II. Mass spectrum (ECNI, methane): molecular anion peak at *m/z* 568 (100%) as well as anion peaks at *m/z* 550 (2%, $[M - H_2O]^-$). ^{1}H NMR (400 MHz): 1.073 [6H, H_3C (16′,17′)]; 1.084 and 1.093 [6H, H_3C(16,17)]; 1.48 [1H, H_{ax}-C(2′)]; 1.50 [1H, H_{ax}-C(2)]; 1.736 [3H, H_3C(18′)]; 1.775 [3H, H_3C(18)]; 1.78 [~1H, H_{eq}-C(2)]; 1.79 [~1H, H_{eq}-C(2′)]; 1.969 [12H, H_3C (19,19′,20,20′)]; 2.05 [1H, H_{ax}-C(4′)]; 2.07 [1H, H_{ax}-C(4)]; 2.38 [1H, H_{eq}-C(4′)]; 2.40 [1H, H_{eq}-C (4)]; 4.01 [2H, H_{ax}-C(3) and H_{ax}-C(3′)]; 6.07 [1H, H-C(10)]; 6.12 [3H, H-C(7), H-C(7′), and H-C (8′)]; 6.16 [1H, H-C(10′)]; 6.25 [2H,H-C(14) and H-C(14′)]; 6.30 [1H, H-C(12)]; 6.36 [1H, H-C (12′)]; 6.64 [2H, H-C(15) and H-C(15′)]; 6.64 [1H, H-C(11′)]; 6.66 [1H, H-C(8)]; 6.74 (1H, H-C(11)]. These chemical shift values were in agreement with previously published values [10].

*13-Zeaxanthin (**10**).* UV–visible absorption: see Tables I and II. Mass spectrum (ECNI, methane): molecular anion peak at *m/z* 568 (100%). ^{1}H NMR (400 MHz): 1.074 [6H, H_3C(16′,17′)]; 1.080 [6H, H_3C(16,17)]; ~1.48 [2H, H_{ax}-C(2) and H_{ax}-C(2′)]; 1.738 [3H, H_3C(18′)]; 1.743 [3H, H_3C(18)]; 1.78 [2H, H_{eq}-C(2) and H_{eq}-C(2′)]; 1.966 [6H, H_3C(19,19′)]; 1.974 [3H, H_3C(20′)]; 1.995 [3H, H_3C(20)]; ~2.04 [2H, H_{ax}-C(4) and H_{ax}-C(4′)]; ~2.39 [2H, H_{eq}-C(4) and H_{eq}-C(4′)]; ~4.00 [2H, H_{ax}-C(3) and H_{ax}-C(3′)]; 6.11 [1H, H-C(14)]; 6.12 [2H, H-C(7′) and H-C(8′)]; 6.14 [2H, H-C(7) and H-C(8)]; 6.16 [1H, H-C(10′)]; 6.20 [1H, H-C(10)]; 6.23 [1H, H-C(14′)]; 6.36 [1H, H-C(12′)]; 6.56 [1H, H-C(15′)]; 6.64 [2H, H-C(11) and H-C(11′)]; 6.79 [1H, H-C(15)]; 6.86 [1H, H-C(12)]. These chemical shift values were in agreement with previously published values [10].

*15Z-Zeaxanthin (**11**).* UV–visible absorption: see Tables I and II. Mass spectrum (ECNI, methane): molecular anion peak at *m/z* 568 (100%). ^{1}H NMR (400 MHz): 1.077 [12H, H_3C (16,16′,17,17′)]; 1.44 [2H, OH-C(3) and OH-C (3′)]; 1.48 [2H, H_{ax}-C(2) and H_{ax}-C(2′)]; 1.744 [6H, H_3C(18,18′)]; 1.775 [2H, H_{eq}-C(2) and H_{eq}-C(2′)]; 1.975 [12H, H_3C(19,19′,20,20′)]; 2.047 [2H, H_{ax}-C(4) and H_{ax}-C(4′)]; 2.39 [2H, H_{eq}-C(4) and H_{eq}-C(4′)]; ~4.00 [2H, H_{ax}-C(3) and H_{ax}-C (3′)]; 6.11 [2H, H-C(7) and H-C(7′)]; 6.137 [2H, H-C(8) and H-C(8′)]; 6.164 [2H, H-C(10) and H-C(10′)]; ~6.40 [2H, H-C(15) and H-C(15′)]; 6.43 [2H, H-C(12) and H-C(12′)]; ~6.66 [2H, H-C(14)

and H-C(14′)]; 6.68 [2H, H-C(11) and H-C(11′)]. These chemical shift values were in agreement with previously published values [10].

Isomerization of all-E-lutein and all-E-zeaxanthin

The geometrical isomers of carotenoids are readily formed by I_2-catalized stereomutation of their all-*E*-isomers [19–21]. In a typical experiment, a solution of lutein or zeaxanthin (50 mg) in benzene under an atmosphere of nitrogen and in the presence of a small crystal of iodine was kept at a short distance from an infrared heating lamp equipped with a 250-W tungsten filament (General Electric, Nela Park, Cleveland, OH,

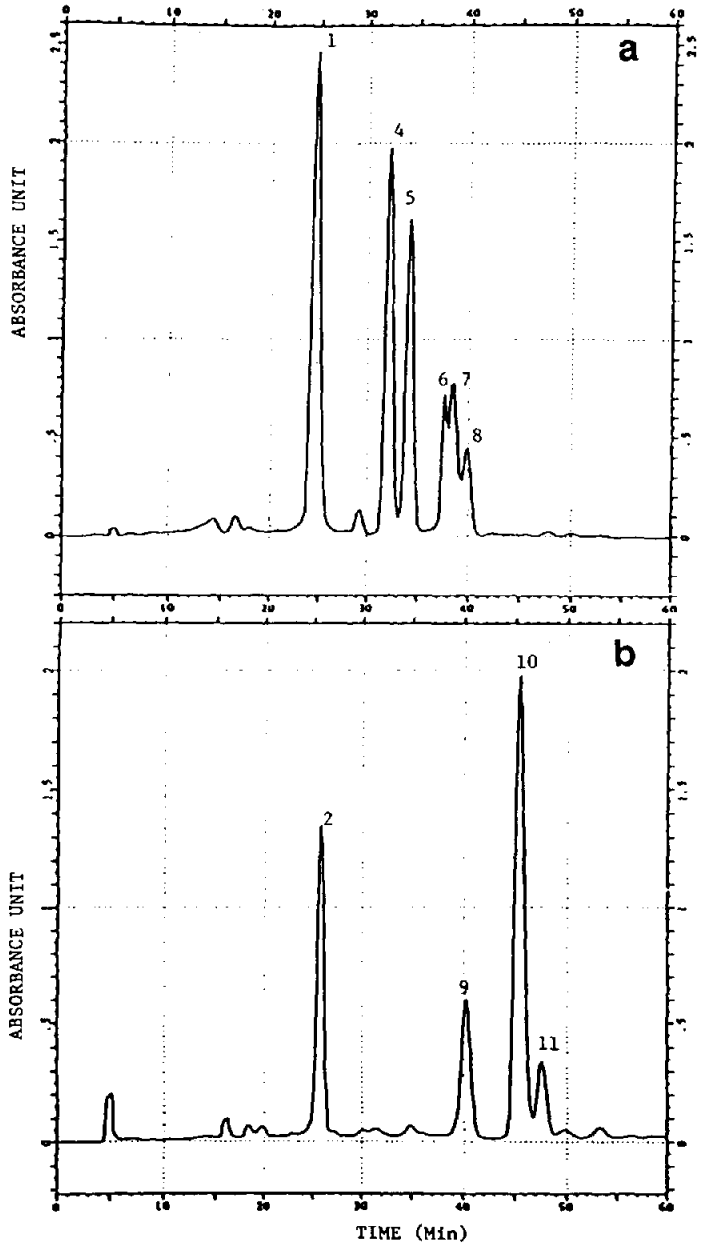


Fig. 3. HPLC profiles (eluent A, conditions described in text) of the geometrical isomers of lutein and zeaxanthin formed by I_2-induced stereomutation of their all-*E* compounds. (a) *Z,E*-Luteins; (b) *Z,E*-zeaxanthins. For peak identification see Fig. 1.

USA) for 1 h. During this time the heat from the infrared lamp resulted in the gentle refluxing of the solutions. Benzene was evaporated under reduced pressure and the crude product was dissolved in eluent A for isolation and purification of the geometrical isomers of lutein and zeaxanthin by semipreparative HPLC.

Lutein. Isomerization of lutein resulted in the formation of six major components which were separated by HPLC (Fig. 3a, eluent A) and identified as: all-*E*-lutein (34%) (peak 1, HPLC retention time 24.70 min), 9*Z*-lutein (24%) (peak 4, HPLC retention time 32.16 min), 9′*Z*-lutein (20.3%) (peak 5, HPLC retention time 34.17 min), 13*Z*-lutein (peak 6, HPLC retention time ~37.80 min), 13′*Z*-lutein (peak 7, HPLC retention time ~38.41) (total of unresolved 13*Z*- and 13′*Z*-lutein = 16%) and 15*Z*-lutein (5.7%) (peak 8, HPLC retention time 39.92 min).

Zeaxanthin. Isomerization of zeaxanthin resulted in the formation of four major components which were separated by HPLC (Fig. 3b, eluent A) and identified as: all-*E*-zeaxanthin (26.6%) (peak 2, HPLC retention time 25.80 min), 9*Z*-zeaxanthin (16%) (peak 9, HPLC retention time 40.5 min), 13*Z*-zeaxanthin (49%) (peak 10, HPLC retention time 45.46 min) and 15*Z*-zeaxanthin (8.4%) (peak 11, HPLC retention time 47.58 min).

RESULTS AND DISCUSSION

The chromatographic profile of the polar carotenoid fraction of human plasma containing dihydroxycarotenoids which were separated by semipreparative HPLC on a nitrile-bonded column is shown in Fig. 2. The detailed isolation of this fraction from extracts of human plasma has been described in our earlier publication [11]. In the order of chromatographic elution, the prominent dihydroxycarotenoids have been identified as all-*E*-(3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol (all-*E*-lutein, peak 1), all-*E*-(3*R*,3′*R*)-β,β-carotene-3,3′-diol (all-*E*-zeaxanthin, peak 2), all-*E*-(3*R*,3′*S*,6′*R*)-β,ε-carotene-3,3′-diol (all-*E*-3′-epilutein, peak 3), 9*Z*-lutein (peak 4), 9′*Z*-lutein (peak 5), 13*Z*-lutein (peak 6), 13′*Z*-lutein (peak 7), 9*Z*-zeaxanthin (peak 9), 13*Z*-zeaxanthin (peak 10) and 15*Z*-zeaxanthin (peak 11). The HPLC separation described in this text does not resolve 13*Z*-lutein (peak 6) and 13′*Z*-lutein (peak 7). Under slightly different HPLC conditions (lower concentrations of methanol and dichloromethane), the separation between these two isomers can be greatly improved. However, the separation of the various geometrical isomers of lutein and zeaxanthin under these improved conditions are quite time-consuming. Therefore in a more practical approach, an HPLC procedure similar to the one described in this text has been employed with an analytical column to determine the combined concentrations of 13*Z*- and 13′*Z*-lutein in human plasma within a reasonable separation time [11]. The absolute configurations of the two prominent hydroxycarotenoids in human plasma, all-*E*-lutein and all-*E*-zeaxanthin, have been determined by ^{1}H NMR and CD as 3*R*,3′*R*,6′*R* and 3*R*,3′*R*, respectively. Therefore the geometrical isomers (*Z*-isomers) of these hydroxycarotenoids would be expected to possess the same configuration as that of their corresponding all-*E*-isomers. The presence of the *Z*-isomers of lutein and zeaxanthin in human plasma is not an artifact of extraction and chromatography. This is due to the fact that samples of all-*E*-lutein and all-*E*-zeaxanthin which were carried through extraction and various chromatographic procedures did not undergo isomerization to their corresponding *Z*-isomers. Additional proof for the presence of the *Z*-isomers of lutein and zeaxanthin were obtained by I_2-induced stereomutation of their all-*E* compounds. The HPLC profiles of an artificial mixture of *Z*,*E*-luteins and *Z*,*E*-zeaxanthins are shown in Fig. 3a and 3b, respectively. Comparison of the HPLC retention times and absorption spectra (Figs. 4 and 5) of the various geometrical isomers of lutein and zeaxanthin obtained by a photodiode-array detector in the HPLC solvents provided additional confirmation for the presence of the *Z*-isomers of these compounds in human plasma. As shown in the HPLC profile in Fig. 2, 15*Z*-lutein is absent in the extracts from human plasma. This isomer of lutein was formed by I_2-in-

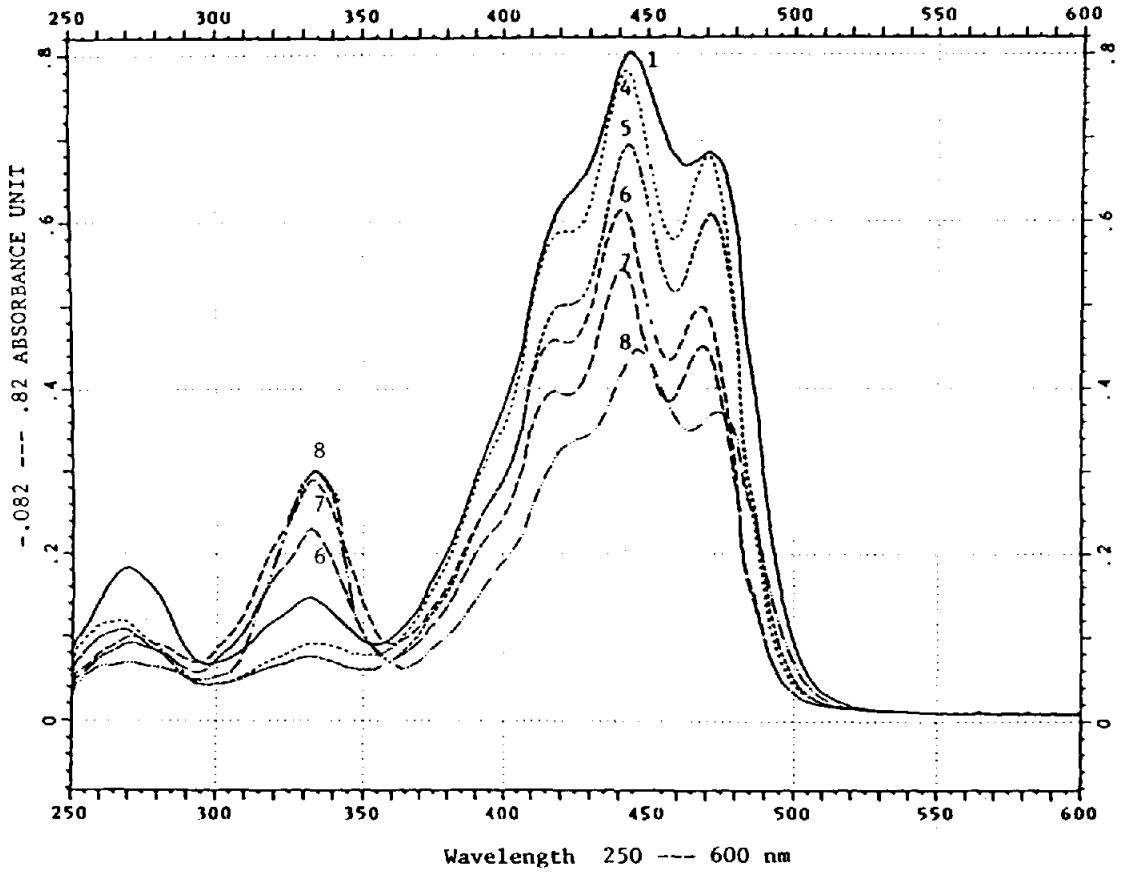


Fig. 4. UV-visible absorption spectra of the geometrical isomers of lutein obtained by a photodiode-array detector in the HPLC solvents (eluent A). 1, all-*E*; 4, 9*Z*, 5, 9′*Z*; 6, 13*Z*; 7, 13′*Z*; 8, 15*Z*. For values of the absorption maxima see Table II.

duced stereomutation of an artificial sample of all-*E*-lutein (Fig. 3a) and has only been tentatively identified from its UV–visible and mass spectra. In an earlier publication, I_2-induced stereomutation of all-*E*-zeaxanthin has resulted in the formation of eleven *Z*/*E*-isomers for zeaxanthin [10]. These were a mixture of mono-*Z*-, di-*Z*- and poly-Z-isomers of zeaxanthin. However, under the conditions reported in this text, the I_2-induced isomerization of lutein and zeaxanthin has been shown to result in the predominant formation of only mono-*Z*-isomers for these compounds. The mono-*Z*-isomers of lutein and zeaxanthin were shown to be quite stable in crystalline forms and in cold solutions (−20°C) and could be readily reverted to the more thermodynamically stable all-*E*-isomers upon reflux in ethanol. Detailed structural elucidation of the various geometrical isomers of lutein and zeaxanthin is discussed in the following.

Discussion of ^1H NMR data

Lutein. The chemical shifts of all-*E*- and *Z*-isomers of lutein are shown in Table III. The coupling constants are not quoted, since they were found in the expected range. In previous publications it has been well established that high-field ^1H NMR is an extremely efficient technique for determining the geometry of the *Z*-isomers of carotenoids [10,17]. The ^1H NMR spectra of more than 40 isomers of carotenoids from seven types of C_{40}-carotenoids [17] and various mono-*Z*-, di-*Z*- and poly-*Z*-isomers of zeaxanthin in a more recent study [10] have been thoroughly examined.

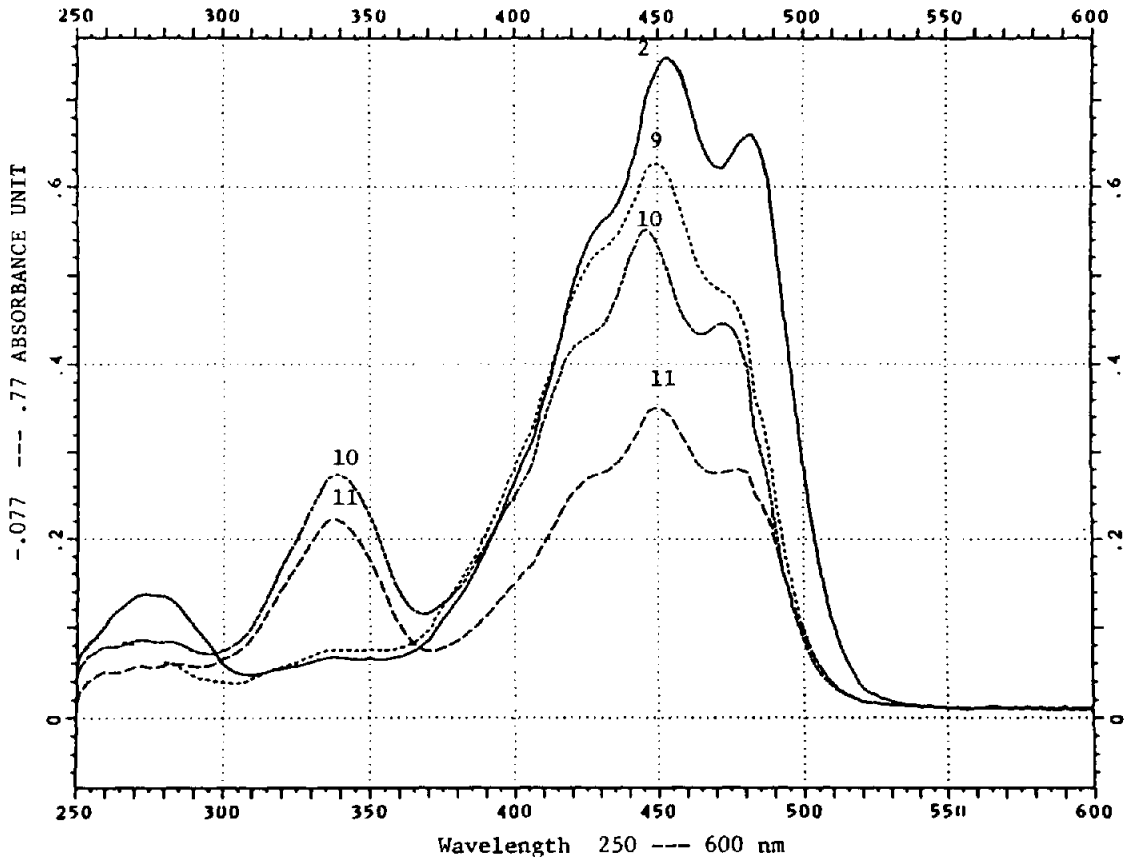


Fig. 5. UV–visible absorption spectra of the geometrical isomers of zeaxanthin obtained by a photodiode-array detector in the HPLC solvents (eluent A). 2, all-*E*; 9, 9*Z*; 10, 13*Z*; 11, 15*Z*. For values of the absorption maxima see Table II.

From the results of these studies it has been well documented that the isomerization shift $\Delta\delta$ (ppm), which is the shift difference $\delta(Z) - \delta(E)$ of the different protons, is quite characteristic and indicative of the stereomutated bond(s). Based on several published ^{1}H NMR values for *Z*-carotenoids, the shift difference $\Delta\delta$ greater than 0.02 ppm can readily assist in the structural elucidation of these compounds [10,17,22–24]. Therefore, the geometrical assignments of the *Z*-isomers of lutein have been based on the relevant $\Delta\delta$ values (>0.02 ppm) listed in Table III. For example in 9*Z*-lutein and 9′*Z*-lutein, the location of the *Z*-double bonds is revealed by a strong downfield shift of H-C(8) (*ca.* 0.55 ppm) in the former and H-C(8′) (*ca.* 0.51 ppm) in the latter, respectively. It is interesting to note that stereomutation at C(9) and even at C(13) results in small but relevant changes in chemical shifts of some of the signals due to the end groups. The same is true for stereomutation at C(9′) and C(13′). This helped to locate the site of stereomutation in the left-hand or right-hand side of the molecule (see Table III and ref. 10). As indicated earlier, 13*Z*-lutein and 13′*Z*-lutein were not separated and the ^{1}H NMR spectrum of the mixture revealed an estimated ratio of approximately 45% for the 13*Z*-isomer and 55% for the 13′*Z*-isomer with an error of $\pm 3\%$. This was estimated from the ratio of the peak intensities at 6.36, near 1.00, and 1.91 ppm. The NMR spectrum of the mixture clearly showed a strong downfield shift of 0.53 ppm for H-C(12) and H-C(12′) in 13*Z*- and 13′*Z*-lutein, respectively. The

chemical shift assignments for the mixture of 13*Z*- and 13′*Z*-lutein listed in Table III are in agreement with those of the pure reference compounds of these two isomeric carotenoids reported in an earlier publication [24]. Based on NMR and CD data, the absolute configuration of all-*E*-lutein isolated from extracts of human plasma has been determined as 3*R*,3′*R*,6′*R*. Although the CD spectra of each of the individually isolated *Z*-isomers of lutein have not been determined, it has been assumed that the absolute configuration of these isomers is the same as the all-*E*-lutein.

3′-Epilutein. The ^{1}H NMR, CD, UV–visible and mass spectral data for 3′-epilutein isolated from human plasma are provided in the experimental section. The relative configuration at C(3′) and C(6′) of 3′-epilutein [(3*R*,3′*S*,6′*R*)-lutein], also known as Calthaxanthin, followed from ^{1}H NMR [17]. Its absolute configuration was determined by comparison of CD and UV–visible data with those previously reported in the literature [14,18,25]. The ^{1}H NMR spectrum of 3′-epilutein [(3*R*,3′*S*,6′*R*)-lutein] showed a broad doublet (J ~9.5 Hz) due to H-C(6′) at *ca.* 2.20 ppm, whereas in (3*R*,3′*R*,6′*R*)-lutein the signal due to this proton is shifted downfield by *ca.* 0.21 ppm and appears at *ca.* 2.41 ppm. Other signals of the two end-groups are also slightly differently shifted (see Table I of ref. 17). Therefore the H-C (6′) and H-C(3′) in this fraction isolated from human plasma are in a *cis*-configuration. Finally, the CD spectrum of this compound in EPA (diethyl ether–isopentane–ethanol, 5:5:2) which is in complete agreement with the reported spectrum for 3′-epilutein establishes the absolute configuration for this compound [14,18,25]. From the ^{1}H NMR spectrum of the isolated 3′-epilutein, it appears that only the all-*E*-isomer is present in the extracts from human plasma. This may be due to the relatively low concentration of this compound in human plasma in comparison with that of *Z*,*E*-lutcins and *Z*,*E*-zeaxanthins. The relative ratio of *Z*,*E*-luteins/*Z*,*E*-zeaxanthins/all-*E*-3′-epilutein = 66%:25%:9% has been calculated from the HPLC profiles of plasma extracts for a number of human subjects [11]. Therefore, the absence of the *Z*-isomers of 3′-epilutein in human plasma may to some extent be related to the low HPLC detection sensitivity for the *Z*-isomers of this compound.

Zeaxanthin. The ^{1}H NMR data for the geometrical isomers of zeaxanthin have been provided in the experimental section. These data are in complete agreement with previously published values [10]. Particularly noticeable signals in the ^{1}H NMR spectra of 9*Z*- and 13*Z*-zeaxanthin are the H-C(8) and H-C(12) which are accompanied by a strong downfield shift of *ca.* 0.5 ppm in comparison with those of all-*E*-zeaxanthin, respectively. In 15*Z*-zeaxanthin, stereomutation can be readily detected from an interchange of the relative position of the signals of H-C(14,14′) and H-C(15,15′) [10]. Based on ^{1}H NMR and CD data of all-*E*-zeaxanthin, the absolute configuration for this compound isolated from human plasma has been established as 3*R*,3′*R*. Although the CD spectra for individually isolated *Z*-isomers of zeaxanthin were not determined in the study reported here, it is reasonable to assume that the absolute configuration for these compounds is the same as their all-*E*-isomer.

Discussion of UV–visible data

Geometrical isomers of lutein. The absorption maxima of the geometrical isomers of lutein and zeaxanthin isolated from an extract of human plasma determined in various solvents are shown in Table I. These values have also been determined by a photodiode-array detector in the HPLC solvents (Table II). The absorption spectra of 9*Z*- and 9′*Z*-lutein in single solvents did not show an intense peak in the near-UV region and exhibited a hypsochromic shift of 4 to 6 nm in comparison to all-*E*-lutein. From the absorption maxima of 9*Z*-lutein and 9′*Z*-lutein listed in Table I as well as the absorption spectra (Fig. 4) obtained by a photodiode-array detector in the HPLC solvents, one can distinguish these compounds from one another. For example, the evaluation of the absorption maxima in HPLC solvents (Table II) reveals hypsochromic shifts of 6 nm for 9*Z*-lutein (HPLC peak 4, λ_{max} = 442 nm) and 4 nm for 9′*Z*-lutein (HPLC peak 5, λ_{max} = 444 nm) from the absorption maximum of all-*E*-

lutein (HPLC peak 1, λ_{max} = 448 nm). Therefore preliminary HPLC peak assignments can be based on the fact that in 9′*Z*-lutein the location of the *Z*-double bond is expected to result in a slightly more conjugated polyene system than that of 9*Z*-lutein. This would in turn result in an increase in the absorption maximum of 9′*Z*-lutein by 2 nm from that of 9*Z*-lutein. As shown in Table I, this difference in main absorption maxima of 9*Z*- and 9′*Z*-lutein in single solvents is about 1 nm. These preliminary structural assignments were later confirmed unambiguously by high-field (400 MHz) ^{1}H NMR spectroscopy.

From the HPLC profiles shown in Figs. 2 and 3a, it is clear that 13*Z*-lutein (peak 6) and 13′*Z*-lutein (peak 7) are coeluting. The absorption maxima of these isomers in single solvents (Table I) and the HPLC solvents (Table II) reveal the presence of a strong *Z*-peak in the near-UV region and a hypsochromic shift of 6 to 8 nm from the absorption maximum of all-*E*-lutein. Since 13*Z*- and 13′*Z*-lutein were isolated as a mixture, the identification of the HPLC peaks (Figs. 2 and 3a) for these compounds were based on their absorption maxima (Fig. 4, Table II) in the HPLC solvents. Owing to the location of the *Z*-double bond in 13′*Z*-lutein, the main absorption maximum in this compound (λ_{max} = 442 nm) appears at a slightly higher wavelength ($\sim$2 nm) than that of 13*Z*-lutein (λ_{max} = 440 nm).

The absorption spectra of 15*Z*-lutein in the HPLC solvents (Table II) and single solvents (Table I) also show an intense *Z*-peak in the near-UV region. The presence of an intense *Z*-peak in the near-UV region is characteristic of 13*Z*- and 15*Z*-carotenoids [10,26]. However, the hypsochromic shift in the absorption maximum of 15*Z*-lutein is between 2 to 4 nm from that of all-*E*-lutein. This can be clearly seen from the absorption spectra of the geometrical isomers of lutein shown in Fig. 4. Although 15*Z*-lutein was not isolated from human plasma, a low concentration of this compound may well be present in human plasma. However, due to HPLC peak overlap between 15*Z*-lutein and 9*Z*-zeaxanthin (Figs. 2 and 3), the detection of this isomer at the present levels in human plasma has not been possible.

Geometrical isomers of zeaxanthin. The absorption maxima of all-*E*-zeaxanthin and its three mono-*Z*-isomers isolated from an extract of human plasma in various solvents and HPLC eluent A are shown in Tables I and II, respectively. The absorption spectra of 9*Z*-zeaxanthin in various solvents did not contain an intense *Z*-peak in the near-UV region and exhibited a hypsochromic shift of 4 to 6 nm from its all-*E* compound. The absorption spectra of both 13*Z*- and 15*Z*-zeaxanthin showed an intense *Z*-peak in the near-UV region. This can be clearly seen from the absorption spectra of the geometrical isomers of zeaxanthin in HPLC solvents shown in Fig. 5. Preliminary assignments for the geometry of these isomers were based on the hypsochromic shift in the absorption maximum of 13*Z*-zeaxanthin (6–8 nm) from its all-*E* compound which was about 4 nm more than the hypsochromic shift for 15*Z*-zeaxanthin (2–4 nm). These assignments were later confirmed unambiguously by high-field (400 MHz) ^{1}H NMR spectroscopy.

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