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Application of HPLC coupled with DAD, APcI-MS and NMR to the analysis of lutein and zeaxanthin stereoisomers in thermally processed vegetables

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Abstract

A method for the simultaneous determination of lutein and zeaxanthin stereoisomers by HPLC-DAD was developed. For this purpose, (*Z*)-isomers of lutein and zeaxanthin were prepared by iodine-catalyzed photoisomerization and their structures elucidated by 1D- and 2D-LC-NMR spectroscopy, by APcI-MS in the positive mode, and by UV/Vis spectroscopy. Near baseline separation was achieved for (13-*Z*)-lutein, (13′-*Z*)-lutein, (*all-E*)-lutein, (9-*Z*)-lutein, (9′-*Z*)-lutein, (13-*Z*)-zeaxanthin, (*all-E*)-zeaxanthin, and (9-*Z*)-zeaxanthin. The influence of selected thermal treatments on degradation and isomerization of lutein and zeaxanthin was assessed. Sweet corn and spinach were sterilized (T_{max} = 121 °C, F = 5) and blanched (t = 2 min, steam), respectively. Heating resulted in decreases in total lutein content in sweet corn by 26% and in spinach by 17%. Total zeaxanthin content in sweet corn decreased by 29%. The amount of (*Z*)-isomers of lutein and zeaxanthin increased in sweet corn from 12% to 30% and 7% to 25%, respectively, whereas in fresh spinach a decrease in lutein (*Z*)-stereoisomers from 21% to 14% was observed.
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1. Introduction

Numerous epidemiological studies suggest that consumption of carotenoids is associated with a lower risk for several types of degenerative diseases in human beings (Schünemann et al., 2002). Carotenoids may protect humans against certain types of cancer, cardiovascular and other diseases associated with ageing (Michaud et al., 2000; Slattery et al., 2000). The dihydroxy carotenoids lutein and zeaxanthin have been identified as the major constituents of the macular pigment of the human retina (Snodderly, 1995). The detection of oxidation products of lutein and zeaxanthin in the human retina supports the hypothesis that dietary lutein and zeaxanthin may act as antioxidants in the macular region (Khachik, Bernstein, & Garland, 1997). Thus, protective activity against the two common eye diseases of ageing, cataract and age-related macular degeneration (AMD), is exhibited (Snodderly, 1995).

Evidence has been provided that supplementation with lutein esters results in an increment of lutein in

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human serum and of optical density in the retina, respectively (Landrum, Bone, & Kilburn, 1997). Furthermore, visual function in patients with age-related cataracts who received lutein supplements improved. This suggests that a higher intake of lutein, through lutein-rich fruit and vegetables or supplements, may have beneficial effects on the visual performance of people with age-related cataracts (Olmedilla, Granado, Blanco, & Vaquero, 2003).

While green leafy vegetables (e.g., spinach, lettuce, kale) are the most important dietary sources of lutein, zeaxanthin is mainly ingested with sweet corn and orange paprika (Holden et al., 1999; Sommerburg, Keunen, Bird, & van Kuijk, 1998). Naturally occurring xanthophylls are usually present in their (*all-E*)-configuration. Except for minimally processed salads and fruits, most of the edible plants are thermally processed prior to consumption. During processing of food such as drying, microwave heating, canning, baking, and cooking, (*all-E*)-carotenoids are partially converted into their (*Z*)-isomers (Chen, Peng, & Chen, 1995; Marx, Schieber, & Carle, 2000, 2002; Pott, Marx, Neidhart, Mühlbauer, & Carle, 2002; van den Berg et al., 2000). Furthermore, storage time and storage conditions, such as temperature and light, facilitate the formation of (*Z*)-isomers (Chen, Peng, & Chen, 1996; Tang & Chen, 2000). The nutritional consequences of *E*/*Z* isomerization are changes in bioavailability and physiological activity (Böhm, Puspitasari-Nienaber, Ferruzzi, & Schwartz, 2002; Castenmiller & West, 1998; Erdmann et al., 1998). Literature data also suggest that each carotenoid shows an individual pattern of absorption, plasma transport, and metabolism. Investigating the effects of processing on carotenoid stability is therefore of great interest.

Despite numerous studies, a complete separation of lutein and zeaxanthin stereoisomers in a single HPLC chromatographic run has not yet been realized (Sander, Sharpless, & Pursch, 2000). Therefore, the objective of the present study was to establish a method for separating the major stereoisomers of lutein and zeaxanthin by HPLC suitable for routine analysis. Unambiguous elucidation of the key stereoisomers by NMR spectroscopy, mass spectrometry and UV/Vis spectra was a further aim of the study.

Since the C_{30} stationary phase used in the present study has successfully been applied to the determination of carotene stereoisomers from carrot juices and for the simultaneous quantification of carotenes and tocopherols from vitamin supplemented drinks (Marx et al., 2000, Marx, Schieber, & Carle, 2002; Schieber, Marx, & Carle, 2002), this analytical system was adapted to the separation of geometrical stereoisomers of lutein and zeaxanthin, and the sample preparation was modified for vegetables.

2. Materials and methods

2.1. Materials

All chemicals used were purchased from VWR (Darmstadt, Germany) and were of reagent grade. HPLC solvents were of gradient grade. (*All-E*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol ((*all-E*)-lutein) and (*all-E*,3*R*,3′*R*)-β,β-carotene-3,3′-diol ((*all-E*)-zeaxanthin) were supplied by Hoffmann-La Roche (Basel, Switzerland). (*Z*)-Isomers were obtained by iodine-catalyzed photoisomerization of the (*all-E*)-carotenoids (Zechmeister, 1962). To avoid degradation and isomerization, amber glass ware was used and processing was developed under dim light conditions. Spinach (*Spinacia oleracea* L.) and sweet corn (*Zea mays* L.) were purchased from a local market in Stuttgart, Germany.

2.2. Preparation of standards

(*All-E*)-lutein and (*all-E*)-zeaxanthin was treated separately by iodine-catalyzed photoisomerization until equilibria mixtures of stereoisomers were obtained. After 5 mg (8.8 μmol) of (*all-E*)-carotenoid was weighed into a 50 mL flask and dissolved in a few drops of toluene, 5 mL of iodine in hexane ($c = 40$ μmol/L) were added (final iodine concentration about 1–2% (w/w) of the carotenoid). The solution was exposed to fluorescent light (polychrome; one fluorescent lamp: L58W/25 universal white and four fluorescent lamps: L36W/76 nature de luxe (Osram, Munich, Germany)) for 30 min at 20 °C. The illumination intensity (3900 lx) was determined with a luxmeter (Mavolux-digital, Gossen, Nuernberg, Germany). After washing the solution twice with $Na_2S_2O_3$ solution (1 mol/L), an aliquot of 5 mL was transferred to another flask for the analytical separation. Both flasks were evaporated in vacuo ($T < 30$ °C). The residue used for the analytical separation was dissolved in 2-propanol and made up to a volume of 5 mL, the residue used for the preparative separation was dissolved in acetone and made up to a volume of 25 mL.

2.3. Chromatography

2.3.1. Analytical chromatography

The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with a system controller SCL-10Avp, an auto injector SIL-10ADvp, a solvent delivery module LC-10Avp with a low pressure mixing valve FCV-10ALvp, a column oven CTO-10Avp, a degasser GT-154, and a diode array detector SPD-10Avp. All data were processed using software Class VP (Version 5.03). The chromatographic analysis was performed using an analytical scale C_{30} reversed phase column

(250 mm × 4.6 mm i.d.) with a particle size of 5 μm (YMC, Wilmington, MA, USA).

2.3.1.1. Solvent system 1. Xanthophylls were eluted isocratically with acetone/water (82/18, v/v) at a column temperature of 20 °C for 45 min. The flow rate was set at 1 mL/min.

2.3.1.2. Solvent system 2. Eluent A consisted of methanol/*tert*-butyl methyl ether (MTBE)/water (92:4:4, v/v), eluent B was prepared by mixing MTBE/methanol/water (90:6:4, v/v). Separation was performed at a column temperature of 20 °C using a linear gradient from 100% A to 6% B within 80 min at a flow rate of 1 mL/min. Aliquots of 20 μL were used for HPLC.

2.3.2. Preparative chromatography

Sample enrichment was carried out on a semipreparative C_{30} reversed phase column (250 mm × 10 mm i.d.). The particle size was 5 μm (YMC, Wilmington, MA, USA). The HPLC system (Bischoff, Leonberg, Germany) consisted of a system controller LC-CaDI 22-14, two solvent delivery modules HPLC compact pump 2250, and a UV–VIS detector SPD-10AVvp (Shimadzu Corporation, Kyoto, Japan). The software McDAcq32 Control (Version 2.0) was from Bischoff (Leonberg, Germany). Chromatographic runs were monitored at 445 nm.

350 μL of the solution of isomerized carotenoids was used for HPLC. Main xanthophyll isomers were eluted isocratically with acetone/water (89:11, v/v) at ambient temperature for 25 min. The flow rate was set at 4 mL/min.

2.4. NMR spectroscopy

LC-NMR measurements were performed on a Varian Unity Inova 500 MHz NMR spectrometer (Darmstadt, Germany) equipped with an ID-PFG probe with a flow cell of 65 μL active volume. Solvent suppression was carried out by WET (Smallcombe, Patt, & Keifer, 1995). The HPLC system consisted of a Varian 9012 pump and a Varian 9050 UV detector. Chromatographic runs were optimized for the best compromise between resolution and maximum signal intensity together with a small peak width which is essential for 2D NMR experiments. Thus, isocratic systems consisting of acetone and varying ratios of D_2O (between 8% and 15%, v/v), depending on the retention times of the individual isomers, were used. The system was operated at ambient temperature with a flow rate of 0.8 mL/min. Monitoring was performed at 445 nm. LC-^{1}H-NMR spectra were recorded in the stop-flow mode with 1000–2000 transients per ^{1}H-NMR spectrum. WETG-COSY spectra were acquired using 48–160 repetitions for 128 or 256 increments and 2K data points with a spectral width of 9000 Hz in both dimensions and a relaxation delay (d_1) of 1 s. ^{1}H chemical shifts were referenced to the residual solvent signal at $\delta = 2.05$ ppm (acetone) relative to TMS.

2.5. HPLC–APcI–MS coupling

LC-MS analyses were performed on an HP 1100 HPLC system (Hewlett Packard, Waldbronn, Germany) coupled to a Micromass VG platform II quadrupole mass spectrometer equipped with an APcI interface (Manchester, UK). The HPLC system was equipped with a HP 1100 autosampler, a HP 1100 thermoregulator, and a HP 1100 diode array detector module. MS parameters: APcI$^+$, temperature 150 °C, probe temperature 400 °C, capillary 3.6 kV, HV lens 0.5 kV. The system was operated in full scan mode (*m/z* 200–800, cone 35 V). The chromatographic conditions corresponded to those of the analytical separation using solvent system 2.

2.6. Quantification

Quantification was performed by UV/Vis spectral analysis using published specific absorption coefficients ($A^{1\%}_{1cm}$) for (*all-E*)-lutein [2550 at 445 nm (ethanol)] and (*all-E*)-zeaxanthin [2540 at 450 nm (ethanol)] (Britton, 1995; Schiedt & Liaaen-Jensen, 1995). Linear calibration graphs based on serial dilutions of stock solutions of (*all-E*)-lutein and (*all-E*)-zeaxanthin. *E*/*Z*-Isomer concentrations were calculated using corresponding (*all-E*)-standard curves.

2.7. Food processing

2.7.1. Sweet corn

Kernels were cut off from the cob with a knife. Silk, husk and extraneous plant material were removed and portions of 240 g of the obtained kernels were filled in lacquered tinplate cans (430 mL) (Schmalbach-Lubeca, Ratingen, Germany). The brine consisted of 40 mL of distilled water containing sodium chloride (1 g/100 mL), sucrose (0.5 g/100 mL) and calcium chloride (160 mg/L). The cans were sealed under nitrogen atmosphere with a vacuum sealing machine (VCV 357, Clemens & Vogl, Braunschweig, Germany). The cans were immediately sterilized (T_{max} = 121 °C, $F = 5$) in a rotary retort (Rotopilot 5, Stock, Neumuenster, Germany). Temperature was measured in the center of the cans. For measuring the effective temperature kernels were speared on the sensors. Sterilized samples were stored in a refrigerator (8 °C) until analysis.

2.7.2. Spinach

Fresh spinach was washed with water and non-edible parts were cut off with a knife. Portions of 200 g were blanched with vapor ($T = \sim 100$ °C) over a covered

water bath (MX 22, Braun, Frankfurt a.M., Germany) for 2 min. For cooling, the spinach was immediately transferred to an ice bath. Inactivation of peroxidase was confirmed by the guaiacol test (data not shown). The spinach was vacuum packed (R 25, Boss, Friedrichsdorf, Germany) and the samples were stored in the dark at −18 °C.

2.8. Preparation of samples

2.8.1. Sweet corn

For homogenization 150 g of chilled sample (8 °C) and 300 mg of pyrogallol as stabilizer were mixed in a blender for 1 min. 50 g of the homogenized sample and 100 g of potassium hydroxide solution (10 g in 100 mL water/methanol, 50:50, v/v) were mixed in an ice-cooled beaker with an ultra-turrax for 2 min. Aliquots of 15 g were transferred to a 100 mL flask. After addition of 25 mL of potassium hydroxide solution (10 g in 100 mL water/methanol, 50:50, v/v) for saponification and 60 mL of light petroleum (40–60 °C) with butylated hydroxytoluene (BHT) (100 mg/L) and butylated hydroxyanisole (BHA) (100 mg/L), the flask was flushed with nitrogen. It was sealed tightly and agitated with a magnetic stir bar for 1 h. The content was once washed in an amber glass separatory funnel with 50 mL of sodium chloride solution (10 g/100 mL) and twice with 50 mL of water to remove alkali. The aqueous layer was reextracted with ethyl acetate until it was colorless. The pooled organic phases were dried with sodium sulfate, evaporated in vacuo ($T < 30$ °C), and the residue dissolved in 2-propanol and made up to a volume of 10 mL.

2.8.2. Spinach

50 g of chilled spinach (8 °C), 100 mL of distilled water and 100 mg of pyrogallol were mixed in a blender for 1 min. About 3 g of the homogenous solution, 30 mL of potassium hydroxide solution (10 g in 100 mL water/methanol, 50:50, v/v) for saponification, and 60 mL of light petroleum (40–60 °C) with BHT (100 mg/L) and BHA (100 mg/L) were put into a 100 mL flask. To ensure an inert atmosphere, the flask was flushed with nitrogen. The sealed flask was agitated with a magnetic stir bar for 1 h. The filtrate obtained by vacuum filtration was transferred to an amber glass separatory funnel. Further steps of the sample preparation corresponded to those described for sweet corn. The residue was dissolved in 2-propanol and made up to a volume of 5 mL.

3. Results and discussion

In the past, C_{18} stationary phases were used for numerous separations, and consequently also for the separation of carotenoids. During the past decade C_{30} stationary phases have turned out to be the best choice for the separation of carotenoids of similar structure (Sander et al., 2000). Recently, the application of a silica-based nitrile-bonded column showed good results for the separation of lutein and zeaxanthin stereoisomers, however, this stationary phase was not able to separate less polar carotenoids (Humphries & Khachik, 2003). Evident advantages of C_{30} stationary phases include enhanced shape selectivity and increased retention, thus allowing the separation of (*all-E*)-lutein and (*all-E*)-zeaxanthin, differing only in the position of one double bond in the ionone ring (Fig. 1).

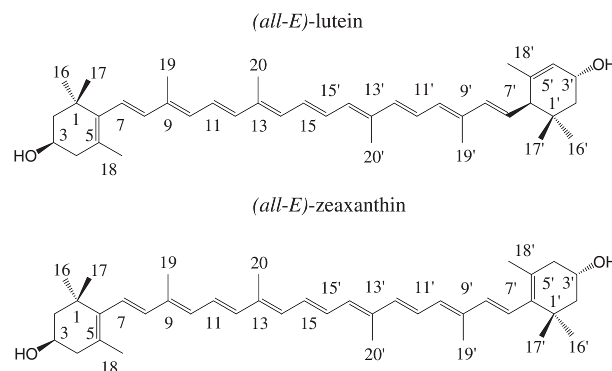

Fig. 1. Structures of (*all-E*)-lutein and (*all-E*)-zeaxanthin.

3.1. Carotenoid standards

The identification and structural elucidation of lutein and zeaxanthin isomers was based on NMR spectra, mass spectra and UV/Vis spectra. After iodine-catalyzed photoisomerization of (*all-E*)-lutein five predominant peaks were observed, while the isomerization of (*all-E*)-zeaxanthin resulted in three major peaks. The separation of a standard mixture of lutein and zeaxanthin stereoisomers using solvent system 1 is shown in Fig. 2. Structure elucidation of the carotenoids was performed by LC-NMR including ^{1}H and WETGCOSY. Thus, the main isomers could be assigned to (13-*Z*)-lutein, (13′-*Z*)-lutein, (*all-E*)-lutein, (9-*Z*)-lutein, (9′-*Z*)-lutein, (13-*Z*)-zeaxanthin, (*all-E*)-zeaxanthin, and (9-*Z*)-zeaxanthin. The NMR data obtained (Table 1) were in good agreement with data from the literature (Dachtler, Glaser, Kohler, & Albert, 2001; Englert et al., 1991) with the exception of the peak assignment of H-4′ ($\delta \approx 5.54$ ppm) of the lutein stereoisomers in the former work. In contrast, in the LC-^{1}H-NMR spectra of (13-*Z*)-, (13′-*Z*)-, (9-*Z*)- and (9′-*Z*)-lutein stereoisomers as well as in the LC-^{1}H-NMR spectrum of the standard (*all-E*)-lutein no signal was detected in the region of $\delta \approx 5.54$ ppm. However, ^{1}H-^{1}H correlation signals between the partially overlapped signal at $\delta = 5.47$ ppm, e.g., in the case of (9-*Z*)-lutein, and $\delta = 1.50$ ppm (Me-

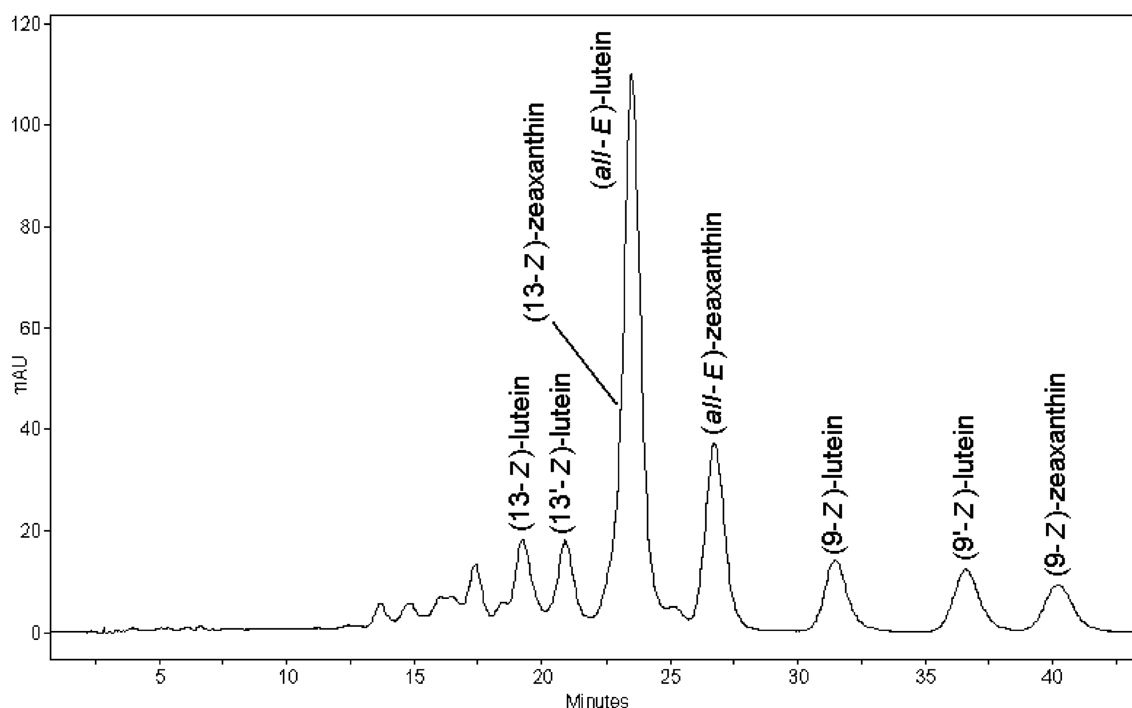

Fig. 2. Separation of a standard mixture of lutein and zeaxanthin stereoisomers (450 nm) by high-performance liquid chromatography using acetone/water as the mobile phase.

Table 1
LC-^{1}H NMR data of the olefinic protons of lutein and zeaxanthin stereoisomers. Acetone/D_2O; ref = 2.05 ppm (acetone); 500 MHz; δ [ppm], mult, *J*[Hz][a]

	Lutein					Zeaxanthin		
	(13-*Z*)	(13′-*Z*)	(*All-E*)	(9-*Z*)	(9′-*Z*)	(13-*Z*)	(*All-E*)	(9-*Z*)
H-4′	5.45, *bs*	5.46, *bs*	5.45, *bs*	5.47, bs	5.48, *bs*			
H-7	6.12, ov[b]	6.10, ov[b]	6.09, ov[b]	6.14, *d*, *J* = 15.4	6.11, ov[b]	6.10, ov[b]	6.11, ov[b]	6.14, ov[b]
H-7′	5.43, *dd*, *J* = 10.2, 15.5	5.45, *dd*, *J* = 10.4, 14.1	5.43, *dd*, *J* = 10.5, 15.8	5.45, *dd*, *J* = 10.3, 15.3	5.47, ov[b], *dd*-like	6.10, ov[b]	6.11, ov[b]	6.11, ov[b]
H-8	6.12, ov[b]	6.12, ov[b]	6.09, ov[b]	6.67, *d*, *J* = 15.8	6.11, ov[b]	6.10, ov[b]	6.11, ov[b]	6.70, *d*, *J* = 16.0
H-8′	6.13, ov[b]	6.09, ov[b]	6.13, ov[b]	6.14, *d*, *J* = 15.5	6.79, *d*, *J* = 15.7	6.10, ov[b]	6.11, ov[b]	6.11, ov[b]
H-10	6.21, *d*, *J* = 11.5	6.15, ov[b]	6.13[c], *d*, *J* ≈ 11.5	6.05, *d*, *J* = 11.4	6.15, *d*, *J* = 11.4	6.22, *d*, *J* = 11.2	6.15, *d*, J = 11.0	6.05, *d*, *J* = 11.5
H-10′	6.12, ov[b]	6.20, *d*, *J* = 12.1	6.10[c], ov[b]	6.11, *d*, *J* ≈ 10.5	6.00, *d*, *J* = 12.2	6.14, *d*, *J* = 11.8	6.15, *d*, *J* = 11.0	6.14, ov[b]
H-11	6.66, *dd*, *J* = 11.6, 14.6	6.65, *dd*, *J* = 9.7, 14.5	6.66[d], *dd*, *J* = 11.4, 14.3	6.77, *dd*, *J* = 12.0, 14.6	6.68, ov[b]	6.67, *dd*, *J* = 11.0, 15.7	6.68, ov[b]	6.77, *dd*, *J* = 11.8, 15.0
H-11′	6.61, *dd*, *J* = 11.4, 15.0	6.63, *dd*, *J* = 9.7, 14.7	6.62[d], ov[b]	6.64, *dd*, *J* = 11.3, 15.1	6.84, *dd*, *J* = 11.5, 14.7	6,66, *dd*, *J* = 11.4, 14.7	6.68, ov[b]	6.67, ov[b]
H-12	6.94, *d*, *J* = 14.6	6.33, *d*, *J* = 15.1	6.34, *d*, *J* = 14.3	6.29, *d*, *J* = 15.6	6.36, *d*, *J* = 15.0	6.95, *d*, *J* = 14.8	6.36, *d*, *J* = 14.7	6.29, *d*, *J* = 15.1
H-12′	6.33, *d*, *J* = 15.0	6.94, *d*, *J* = 15.1	6.34, *d*, *J* = 14.3	6.34, *d*, *J* = 15.1	6.28, *d*, *J* = 14.7	6.34, *d*, *J* = 14.8	6.36, *d*, *J* = 14.7	6.35, *d*, *J* = 15.0
H-14	6.10, ov[b]	6.23, *d*, *J* = 11.9	6.25, ov[b]	6.26, ov[b]	6.24, ov[b]	6.11, ov[b]	6.27, ov[b]	6.26, ov[b]
H-14′	6.23, *d*, *J* = 11.4	6.10, ov[b]	6.25, ov[b]	6.26, ov[b]	6.24, ov[b]	6.24, *d*, *J* = 12.2	6.27, ov[b]	6.26, ov[b]
H-15	6.85, *pt*, *J* = 12.7	6.57, *pt*, *J* = 12.2	6.64, ov[b]	6.65, ov[b]	6.67, ov[b]	6.87, *pt*, *J* = 13.1	6.66, ov[b]	6.66, ov[b]
H-15′	6.57, *pt*, *J* = 12.8	6.85, *pt*, *J* = 12.9	6.64, ov[b]	6.65, ov[b]	6.67, ov[b]	6.59, *pt*, *J* = 13.0	6.66, ov[b]	6.66, ov[b]

[a] Observed coupling constants were not averaged. Assignments based on wetgcosy.
[b] ov: overlapped by other signals.
[c,d] Assignments may be interchanged.

18′) as well as $\delta = 4.12$ ppm (H-3′, bs) which correlates to further protons in the alicyclic region ($\delta = 1.71$ ppm, *dd*, $J = 6.1$, 12.2; and 1.27 ppm, overlapped; $2 \times$ H-2′) indicated that this proton had to be assigned as H-4′. H-7′ was identified by its ^{1}H–^{1}H correlation signals to H-8′ (Table 1) and H-6′ ($\delta = 2.36$ ppm, *bd*, $J = 10.3$). Analogous results were obtained for the remaining lutein stereoisomers. Beside the unambiguous differentiation of lutein and zeaxanthin isomers by LC-NMR (Dachtler et al., 2001), another NMR spectroscopic feature within the (mono-*Z*)-lutein stereoisomers was found that may be used for the rapid assignment of an isomerization site close to the ε- or β-end group under LC-NMR conditions. Comparison of the chemical shift difference of H-4′ and H-7′ (Fig. 3) suggests an isomerization site close to the β-end group in the case of $\Delta\delta = 0.02$ ppm and close to the ε-end group in the case of $\Delta\delta = 0.01$ ppm.

Compared to solvent system 1 consisting of acetone and water (Fig. 2), the separation of xanthophyll stereoisomers using solvent system 2 (MeOH, TBME and water) provided satisfactory results (Fig. 4), since an improved separation especially of (13-*Z*)-zeaxanthin from (*all-E*)-lutein was obtained. As can be seen, this analytical system allowed the separation of (13-*Z*)-lutein, (13′-*Z*)-lutein, (*all-E*)-lutein, (9-*Z*)-lutein, (9′-*Z*)-lutein, (13-*Z*)-zeaxanthin, (*all-E*)-zeaxanthin, and (9-*Z*)-zeaxanthin within 80 min. Identical isomers were reported in extracts of the human retina (Khachik et al., 1997). Apart from these major stereoisomers, several compounds not yet identified were also detected. Although a number of methods for the separation of xanthophyll stereoisomers have been reported, the complete simultaneous separation of *E*/*Z* isomers of lutein and zeaxanthin has not been achieved so far (Dachtler, Kohler, & Albert, 1998; Emenhiser, Sander, & Schwartz, 1995; Emenhiser, Simunovic, Sander, & Schwartz, 1996). In other studies some compounds were only tentatively identified (Delgado-Vargas & Paredes-Lopez, 1997; Emenhiser et al., 1995). It is of particular importance that the method presented allows the separation of (13-*Z*)-zeaxanthin from (*all-E*)-lutein since (13-*Z*)-carotenoids are predominantly formed by thermal treatment (Chen et al., 1995).

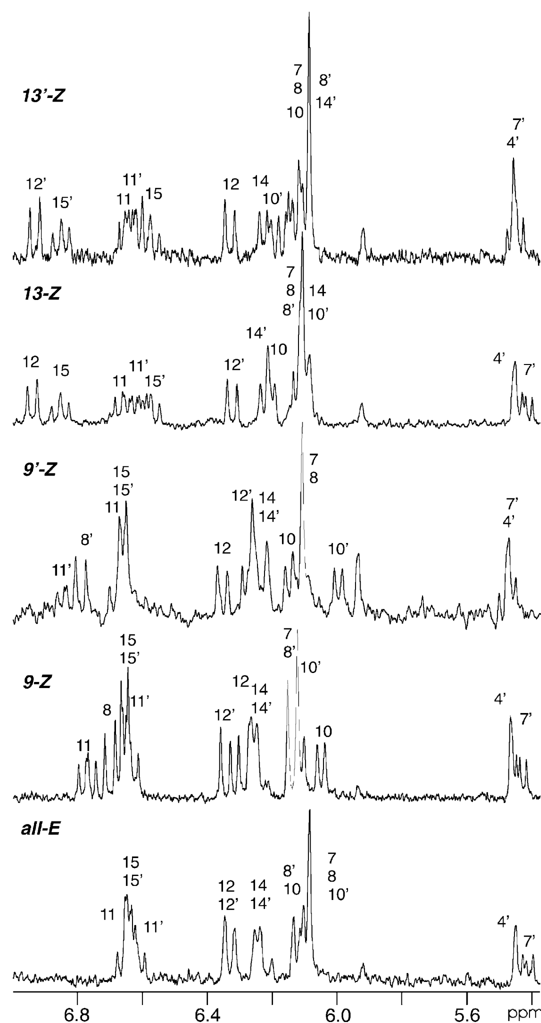

Fig. 3. LC-^{1}H NMR spectra (olefinic region) of (*all-E*)- and four (mono-*Z*)-lutein stereoisomers measured in acetone/D_2O.

Despite the structural similarity of lutein and zeaxanthin, differentiation between both xanthophylls using mass spectrometry was achieved. The mass spectra of the isomers of zeaxanthin showed a base peak at *m*/*z* 569.3, corresponding to $[M + H]^+$ of lutein (Lacker, Strohschein, & Albert, 1999). Due to the different position of the double bond in the ionone ring, (*all-E*)-lutein and its (*Z*)-isomers possess an allylic hydroxyl group at C-3′, which can easily be eliminated, resulting in a base peak at *m*/*z* 551.3 $[M + H - H_2O]^+$.

All UV/Vis spectra were characteristic of lutein and zeaxanthin with respect to the fine structure (400–500 nm) (Table 2). The values given were determined by photodiode-array detection in the corresponding HPLC solvents. The spectrum of (*all-E*)-lutein has typical absorption maxima. The absence of near-UV (about 330 nm) absorbance is characteristic of the *all-E* arrangement of the double bonds. Both pairs of isomers, i.e., (13-*Z*)- and (13′-*Z*)-lutein, and (9-*Z*)- and (9′-*Z*)-lutein, exhibit nearly identical absorption maxima. In general, a small hypsochromic shift is observed with the introduction of a *Z*-bond compared to the *all-E* configuration (Zechmeister, 1962). In comparison to the absorption maxima of (*all-E*)-lutein, (9-*Z*)-lutein and (9′-*Z*)-lutein showed hypsochromic shifts of 5 nm and 4 nm, and (13-*Z*)-lutein and (13′-*Z*)-lutein showed shifts of 8 and 6 nm, respectively.

Beside the differentiation based on typical absorption maxima, a distinction of (*Z*)-isomers is possible owing to the "*cis* peak" between 320 and 380 nm (Zechmeister, 1962), because absorbance around 330 nm generally increases as the position of the *Z*-bond approaches the center of the conjugated system. Strong absorbance in

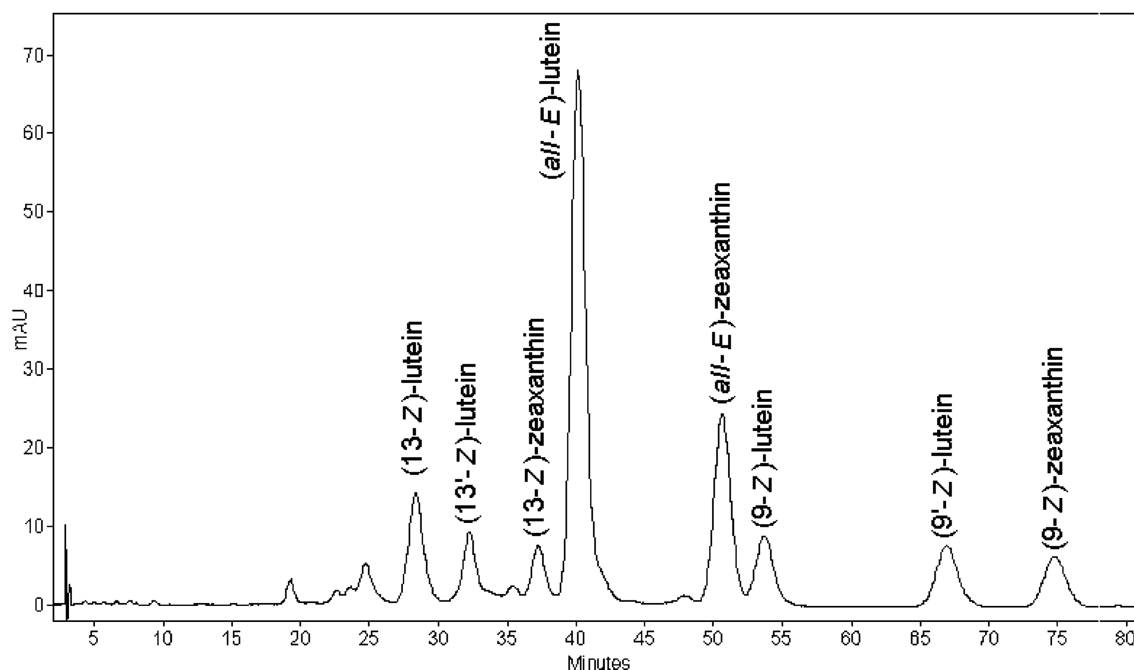

Fig. 4. Separation of a standard mixture of lutein and zeaxanthin stereoisomers (450 nm) by high-performance liquid chromatography using TBME/methanol/water as the mobile phase.

Table 2
Electronic absorption maxima of the predominant isomers of lutein and zeaxanthin

Isomer	Absorption maxima (nm)[a]	$[\varepsilon_2/\varepsilon_1]$[b]	[100III/II][c]
(13-*Z*)-Lutein	330, (417), **436**, 463	0.441	31
(13′-*Z*)-Lutein	331, (417), **438**, 465	0.412	43
(*All*-*E*)-Lutein	332, (423), **444**, 472	0.049	65
(9-*Z*)-Lutein	332, (418), **439**, 467	0.109	67
(9′-*Z*)-Lutein	327, (420), **440**, 468	0.141	50
(13-*Z*)-Zeaxanthin	338, (422), **443**, 470	0.490	10
(*All*-*E*)-Zeaxanthin	339, (428), **450**, 477	0.059	33
(9-*Z*)-Zeaxanthin	338, (421), **445**, 472	0.140	32

Determined in the HPLC eluent (MTBE/methanol/water) employing the photodiode array detector.
[a] Values in bold face represent the main maximum. Values in parentheses mark points of inflection.
[b] Ratio of the absorption intensity ε_2 at the near-UV maximum (327–339 nm) to the absorption intensity ε_1 at the main absorption maximum (436–450 nm).
[c] Peak ratio 100III/II.

this region can be observed for (13-*Z*)- and (13′-*Z*)-lutein and (13-*Z*)-zeaxanthin. This absorbance is reflected in the proportion of $\varepsilon_2/\varepsilon_1$ which is much greater than that of the (*all-E*)- and (9-*Z*)-isomers, as reported by others (Delgado-Vargas & Paredes-Lopez, 1996) (Table 2). A plausible explanation for this is the extinction coefficient depending on the dipole moment in the molecule, which is in turn related to the perpendicular distance between the *Z*-bond and an imaginary line joining the ends of the carotenoid molecule (Zechmeister, 1962). The findings correspond with other published data (Khachik et al., 1992).

An ultimate factor for the differentiation of the fine structure is the ratio between III/II, where the peak height of the most bathochrome absorption band is designated as III and that of the middle absorption band (usually λ_{max}) as II. The baseline or zero value is taken as the minimum between the two peaks. The spectral fine structure is then expressed as a percentage of the ratio of the peak heights III/II. Thus, (*all-E*)-lutein and (*all-E*)-zeaxanthin give % III/II values in ethanol of 60 and 26, respectively (Britton, 1995). An indication of the relationship between spectral fine structure and % III/II can be taken from the spectra (Table 2). In the present study the following ratios were found: 65 for (*all-E*)-lutein, 67 for (9-*Z*)-lutein, 50 for (9′-*Z*)-lutein, 31 for (13-*Z*)-lutein, and 43 for (13′-*Z*)-lutein. The isomers of zeaxanthin followed the same pattern, i.e., 33 for (*all-E*)-zeaxanthin and 32 for (9-*Z*)-zeaxanthin. The smallest ratio of 10 was observed for (13-*Z*)-zeaxanthin.

3.2. Food samples

The analytical system was used to assess the influence of thermal processing of vegetables on the stability and *E*/*Z* isomerization of (*all-E*)-lutein and (*all-E*)-zeaxanthin. Therefore, spinach as an important source of lutein and sweet corn as a rich source of zeaxanthin and lutein were selected. Sweet corn was canned according to the Codex standard (FAO & WHO, 1981). The amount of brine used was less than 20% of the total weight of the product and the cans were sealed under vacuum in a nitrogen atmosphere to avoid oxidation of carotenoids. Spinach is usually processed into frozen products. Prior to freezing, fresh spinach is blanched to inactivate deteriorating enzymes, especially lipoxygenases. Since peroxidase is thermally more stable than lipoxygenases, the absence of peroxidase is considered an indicator for sufficient blanching conditions. By vapor blanching at ~100 °C complete peroxidase inactivation was achieved after 1 min, as determined by the guaiacol test. For the determination of carotenoids all samples were subjected to saponification to remove chlorophylls and lipids, respectively, which might affect chromatographic performance. In contrast to literature data, carotenoids were monitored at their spectral maxima in the mobile phase.

The separation of lutein and zeaxanthin stereoisomers extracted from sterilized sweet corn and blanched spinach is shown in Figs. 5 and 6, respectively. Apart from predominant (*all-E*)-lutein and (*all-E*)-zeaxanthin in the sweet corn extract, (9-*Z*)- and (13-*Z*)-isomers were detected, with (13-*Z*)-, (13′-*Z*)-lutein and (13-*Z*)-zeaxanthin being present in higher amounts (Fig. 5). While in spinach (*all-E*)-zeaxanthin was only found in trace amounts, (*all-E*)-lutein was shown to be the predominant xanthophyll. Consequently, only (*Z*)-isomers of the latter were detected in blanched spinach (Fig. 6).

Total lutein and total zeaxanthin of sterilized samples of sweet corn differed greatly from the corresponding unheated vegetable. Processing resulted in 26% and 29% decreases in total lutein content and zeaxanthin content, respectively. Compared to the control, blanched spinach showed a 17% reduction in total lutein content. Considering the lower temperature and the much shorter heat exposure of blanching compared to sterilization, a substantial part of lutein was degraded.

Heating exerted an additional effect on isomerization since thermal processing of sweet corn was associated with an increase in total amount of (*Z*)-isomers from 12% to 30% and 7% to 25% for lutein and zeaxanthin, respectively (Table 3). Remarkably, the total content of (*Z*)-lutein isomers in fresh spinach was relatively high (21%), but decreased after thermal treatment (14%).

With respect to individual stereoisomers, thermal treatment of sweet corn caused the largest increment of contents of (13-*Z*)-, (13′-*Z*)-lutein and (13-*Z*)-zeaxanthin (11%, 10% and 21%). Since (13-*Z*)-carotenoids are predominantly formed by thermal treatment, these findings correspond with published data (Chen et al., 1996) and can be attributed to the canning process. In contrast, the amount of lutein (*Z*)-stereoisomers of processed spinach did not increase. While the content of (9-*Z*)- and (9‘-*Z*)-lutein decreased, (13-*Z*)- and (13′-*Z*)-lutein virtually remained unaffected. Moreover, since

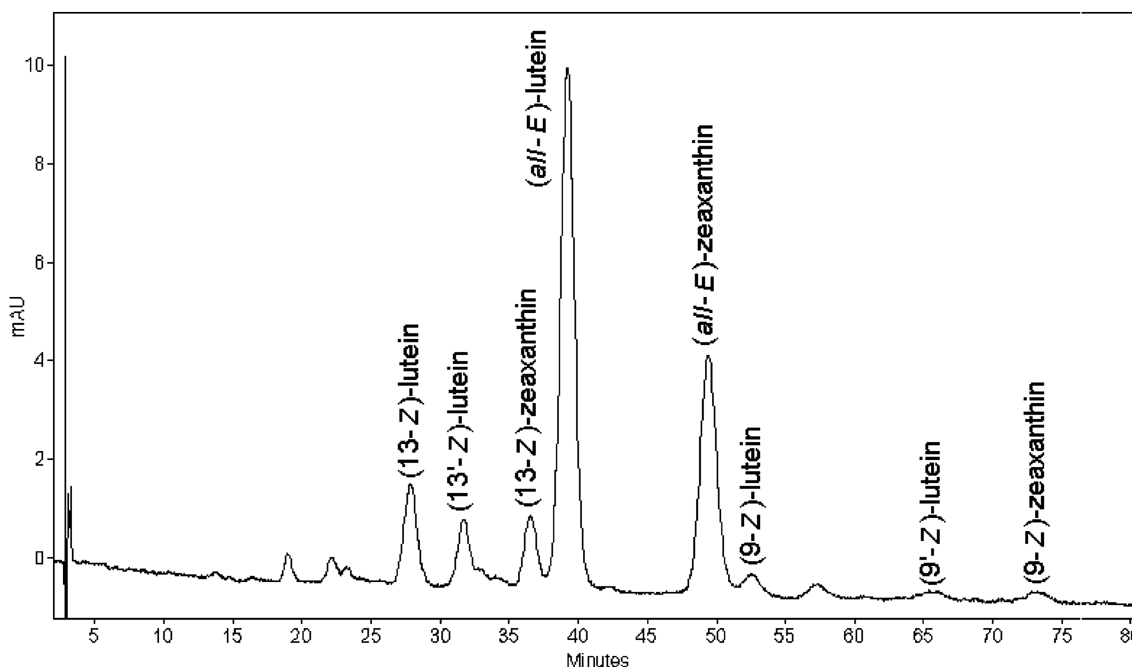

Fig. 5. Separation of lutein and zeaxanthin stereoisomers (450 nm) extracted from canned sweet corn.

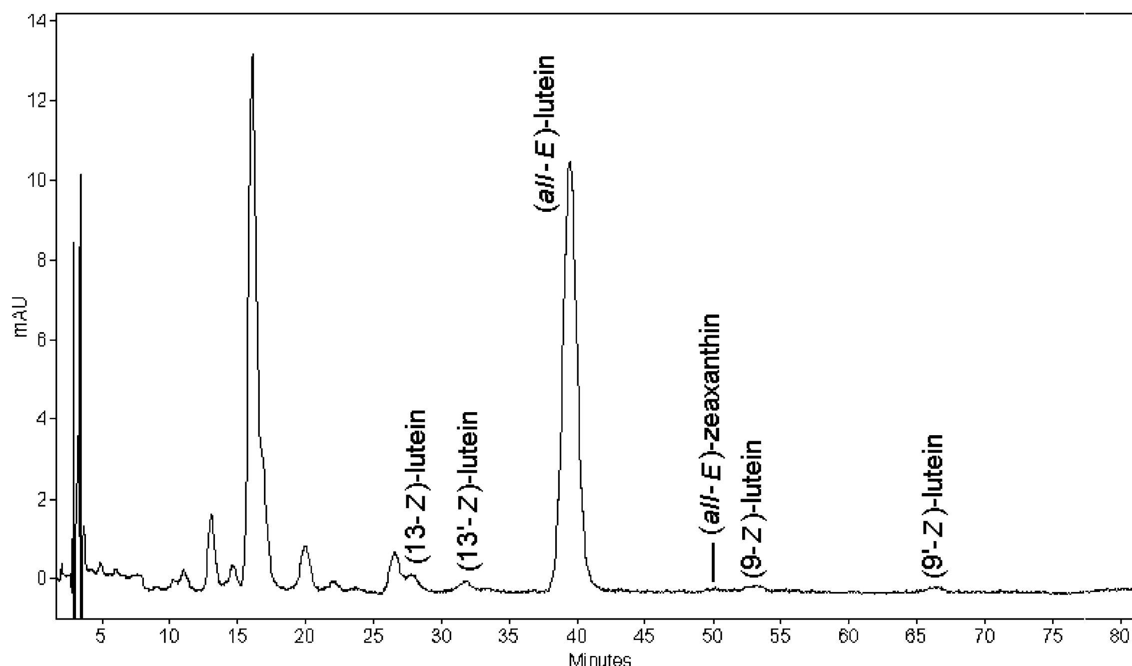

Fig. 6. Separation of lutein and zeaxanthin stereoisomers (450 nm) extracted from blanched spinach leaves.

Table 3
Lutein and zeaxanthin content (mg/100 g dry weight) of raw and heated vegetables

Isomer	Sweet corn		Spinach	
	Raw	Sterilized	Raw	Blanched
(*All-E*)-Lutein	2.07	1.21	34.9	31.6
(13-*Z*)-Lutein	0.05	0.19	3.0	2.4
(13′-*Z*)-Lutein	0.08	0.18	1.3	1.4
(9-*Z*)-Lutein	0.10	0.10	4.1	1.0
(9′-*Z*)-Lutein	0.04	0.05	1.0	0.6
Total lutein	2.34	1.72	44.3	36.9
(*All-E*)-Zeaxanthin	0.86	0.49	tr.[a]	tr.
(13-*Z*)-Zeaxanthin	0.04	0.14	n.d.[b]	n.d.
(9-*Z*)-Zeaxanthin	0.02	0.02	n.d.	n.d.
Total zeaxanthin	0.92	0.65	tr.	tr.

[a] tr.: trace.
[b] n.d.: not detectable.

(9-*Z*)- and (9′-*Z*)-lutein were detected in both unprocessed vegetables and (9-*Z*)-zeaxanthin was detected in fresh sweet corn, the present findings do not confirm previously published studies stating that (9-*Z*)-stereoisomers of lutein and zeaxanthin in sweet corn and spinach could only be detected after thermal treatment (Updike & Schwartz, 2003).

However, the difference of xanthophyll isomerization in corn and spinach upon thermal treatment is not understood so far. It may be assumed that localization of carotenoids in different plastids of vegetables plays an important role. As previously shown, diverging results observed for β-carotene degradation in carrots and mango may be attributed to the deposition of carotenes in different types of chromoplasts (Marx et al., 2000, 2002; Pott et al., 2002). While in sweet corn lutein and zeaxanthin are localized in chromoplasts, lutein as an accessory pigment in photosynthesis is exclusively localized in the chloroplasts in spinach. Chlorophyll derivatives act as sensitizers in the photoisomerization of (*all-E*)-β-carotene. (*All-E*)-β-carotene added to chlorophyll derivatives was photoisomerized (O'Neil & Schwartz, 1995). The influence of thermal treatment of (*all-E*)-carotenoids sensitized by chlorophyll on degradation and isomerization is still unknown.

4. Conclusions

A method for the simultaneous separation of the two xanthophylls (*all-E*)-lutein and (*all-E*)-zeaxanthin including their major (*Z*)-stereoisomers is presented.

All compounds were baseline separated and unambiguously identified by NMR spectroscopy. Using this analytical system investigations on the effects of processing on *E*/*Z* isomerization of carotenoids in sweet corn and spinach showed differences in the extent of degradation and isomerization. Thermal treatment of sweet corn and spinach resulted in a decrease in total amount of lutein and zeaxanthin. Compared to intense heating during the canning process of sweet corn, the moderate blanching of spinach caused a relatively high lutein degradation. Whether temperature may induce isomerization reactions of (*all*-*E*)-lutein sensitized by chlorophyll is a subject of current research. Furthermore, in times of an expanding market for dietary supplements and functional foods, reliable information on carotenoid contents in such products is urgently needed. The analytical method present here may be a useful tool for the food industry and the Food Inspection Board to assess the specification of marketed products.

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