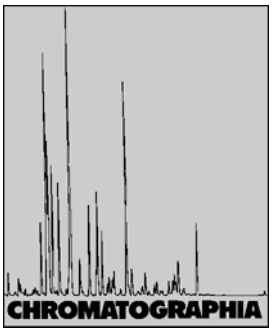
Qualitative and Quantitative Determination of Carotenoid Stereoisomers in a Variety of Spinach Samples by Use of MSPD Before HPLC-UV, HPLC-APCI-MS, and HPLC-NMR On-Line Coupling



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In Memoriam Professor Ernst Bayer

Key Words

Column liquid chromatography-NMR
Matrix solid-phase dispersion
HPLC-APCI-MS
Carotenoid stereoisomers
Quantification
Spinach

Summary

The carotenoids lutein and zeaxanthin have been identified as the macular pigments of the human retina. Nutritional epidemiological reports indicate that high consumption of fruits and vegetables rich in these carotenoids is correlated with reduced risk of some illnesses, e.g. age-related macular degeneration (AMD).

Because carotenoids are extremely sensitive to UV light and oxygen, they occur in nature as several *Z*/*E* stereoisomers which can differ considerably in their biological effectiveness. With particular regard to dietary supplementation we have focussed on identification and quantification of all the carotenoid stereoisomers occurring in a variety of raw and processed home-grown and commercial spinach samples, a natural source of carotenoids. Isolation of the unstable carotenoid stereoisomers from biological tissues without sample-preparation artifacts requires a mild, rapid, complete, and reproducible extraction technique such as matrix solid-phase dispersion (MSPD). Separation and unequivocal structural elucidation of the main carotenoid stereoisomers was achieved by use of hyphenated analytical techniques and exclusion of light and oxygen. HPLC analysis with highly selective C_{30} columns was used for quantitative determination of the main *Z*/*E* carotenoid stereoisomers and HPLC-APCI-MS and HPLC-NMR on-line coupling was used for unequivocal structural elucidation. Whereas HPLC-APCI-MS can distinguish between the carotenoids lutein and zeaxanthin, HPLC-NMR enables identification of all the main *Z*/*E* stereoisomers.

Introduction

Quantitative determination of carotenoid stereoisomers is becoming of increasing interest to the analytical chemist as an increasing number of protective effects are ascribed to carotenoids. Hyphenated analytical techniques such as HPLC-NMR on-line coupling enable unambiguous identification of these stereoisomers and, therefore, offer new possibilities in nutritional and clinical research. Precise analysis, without production of artifacts, is a prerequisite, because the carotenoids are extremely unstable, owing to their antioxidant status, and are susceptible to isomerization and oxidation. Carotenoids therefore occur in nature as many *Z*/*E* stereoisomers which can differ substantially in their biochemical activity [1].

Fruits and vegetables are the most important source of carotenoids in the human diet. For example, lutein and zeaxanthin, which can be found in many dark-green, leafy vegetables such as spinach, broccoli, and kale, are not produced in the human body and must therefore be obtained solely through dietary sources [2, 3]. The bioavailability of the carotenoids in these natural sources is important, because it can vary from one carotenoid to another and even from one stereoisomer to another. Recent studies have revealed that the bioavailability of β-carotene from spinach is low, and highly dependent on the food matrix. The bioavailability of lutein was, in contrast, much higher and less dependent on the food matrix [4, 5]. The determination of *Z*/*E* stereoisomers is important because, for instance, the bioavailability of the *Z* isomers of lycopene is higher than that of all-*E* lycopene both in-vitro and in-vivo [6].

The biochemical relevance of these compounds is given in a recent paper

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which ascribes to the carotenoids protective effects against many diseases, for example cataracts [7], lipid peroxidation [8], coronary heart diseases [9], cancer [9], and age-related macular degeneration (AMD) [9, 10]. The protective effect against AMD is a result of the occurrence of lutein and zeaxanthin in the central yellow spot of the retina, the so-called macula lutea [11, 12]. These macular pigments act as optical filters and protect the retina against phototoxic damage by absorption of blue light, which is not stopped by the cornea and lens and which can cause free-radical damage. They also act as antioxidants in quenching singlet oxygen and scavenging free radicals [13]. A variety of *Z*/*E* stereoisomers have been detected in the retina, and species-specific patterns have been observed [14, 15].

With demonstration of a correlation between dietary carotenoid intake and macular pigment density, the search for nutritional supplements and dietary preventives against AMD, so-called functional food, is becoming increasingly important [16]. Clinical and nutritional studies report that intake of pure lutein and zeaxanthin, and of functional food containing these phytonutrients, is associated with a lower risk of AMD [7, 17, 18]. All these studies have dealt only with the relationship between AMD and the carotenoids lutein and zeaxanthin, however, and none of the studies pertained to stereoisomers. The best possible medicinal or dietary treatment of AMD requires an understanding of biochemical changes in the retinal carotenoid pattern. It is, therefore, necessary to obtain information about the structure and the concentration of every carotenoid stereoisomer in dietary sources of carotenoids, the functional food.

Extraction is the most time-consuming step in the analysis of biological samples, and the source of most errors. Prerequisites for artifact-free isolation of light- and oxygen-sensitive carotenoid stereoisomers is the use of a mild and rapid extraction technique. MSPD combines these prerequisites and is also suitable for quantitative analysis. MSPD also enables extraction directly from solid or viscous samples [19, 20]. Because the extraction procedure takes 20 min only and the carotenoids elute in a highly concentrated fraction of approximately 300 µL, MSPD is the method of choice for artifact-free and quantifiable isolation of carotenoids from biological matrices.

Separation and identification of carotenoid stereoisomers is possible by use of C_{30} stationary phases, because of their greater shape selectivity and higher loading capacity in comparison with C_{18} columns [21]. The higher shape selectivity of C_{30} phases has been characterized by NMR studies and applied to several separation problems [22–24]. To enable unequivocal structural elucidation of all the main carotenoid stereoisomers the use of hyphenated analytical techniques is a prerequisite. The characteristic retention time of each carotenoid stereoisomer, using HPLC-UV detection at 450 nm, can be used for identification purposes by comparison with those obtained from external standard test mixtures. In the analysis of biological tissues, however, matrix effects lead to deviations in retention times.

HPLC-APCI-MS enables assignment of the different carotenoids within one chromatographic run [25–27]. Positive-ion APCI-MS also enables differentiation of structurally similar carotenoids, e.g. the macular pigments lutein and zeaxanthin, in the low pmol range [15]. It is, however, impossible to distinguish between the *Z* and *E* stereoisomers of a carotenoid by use of APCI-MS or APCI-$MS^{(n)}$, because their fragmentation patterns are identical.

On-line HPLC-NMR coupling provides all the structural information needed for unambiguous identification of carotenoid stereoisomers. Because C_{30} stationary phases have a high loading capacity and shape selectivity, they are primarily used for HPLC-NMR coupling experiments [28]. This has been demonstrated by analysis of carotenoid stereoisomers, vitamin E isomers, and flavonoid derivatives [29–31].

This article describes the qualitative and quantitative determination of carotenoid *Z*/*E* stereoisomer patterns in a variety of raw and processed spinach samples. The combined use of MSPD and HPLC with C_{30} stationary phases hyphenated with different detection techniques is necessary to identify all main carotenoid stereoisomers.

Experimental

Preparation of Standards

Pure standards (4 mg) of all-*E* lutein (gifts from Kemin, Des Moines, IA, USA and F. Hoffmann-LaRoche, Basel, Switzerland), all-*E* zeaxanthin, and all-*E* β-carotene (kindly provided by BASF, Ludwigshafen, Germany) were dissolved separately in acetone (2 mL). Iodine-catalyzed isomerization was performed by the method described by Zechmeister [32]. After exposure to sunlight for several hours the standards were stored below –40 °C.

Sample Preparation and Extraction

The spinach samples investigated included a commercial spinach product bought at a local supermarket and two homegrown spinach samples with different growing times (harvested after either 5 or 8 weeks growth). The homegrown spinach was harvested on the day of analysis and prepared for analysis in the same way as it is prepared for consumption. Because MSPD requires homogeneous sample tissue, spinach leaves were washed with deionized water, drained, and chopped into pieces as small as possible. The leaves were then deep frozen in liquid nitrogen and ground with a pestle. All spinach samples were analyzed both raw and after cooking in a microwave oven for 6 min at 600 W with addition of a small amount of water.

MSPD was performed as described by Barker [19]. Spinach (0.5 g) was mixed with MSPD C_{30} sorbent material (30–50 µm, 1.5 g; Bischoff Chromatography, Leonberg, Germany) and few crystals of BHT (butylated hydroxytoluene; Sigma, Steinheim, Germany) as stabilizer. For quantification a known amount of the internal standard β-*apo*-8′-carotenal (Fluka, Buchs, Switzerland) was added to the material in the mortar. The solid mixture was gently blended until a free-flowing powder was obtained; it was then packed into an SPE column and finally compressed with a syringe plunger. After insertion of an end frit the compact column bed was conditioned with water (15 mL) and polar impurities were eluted with another 5 mL of water-methanol mixture. After drying of the column the carotenoids were eluted with acetone until the extract became colorless (less than 500 µL). Saponification of the chlorophylls was not necessary because the chlorophylls do not disturb carotenoid stereoisomer separation and saponification leads to degradation of the carotenoid content [33].

Chromatography

HPLC was performed with an HP 1100 system equipped with a G1312A binary pump and a G 1314A UV detector (Agilent Technologies, Waldbronn, Germany) and controlled by HyStar software (Bruker Daltonics, Bremen, Germany). All separations were performed on a ProntoSIL C_{30} column (3 μm particle size, 200 Å pore diameter, 250 mm × 4.6 mm) kindly provided by Bischoff Chromatography.

Gradient elution with a binary mixture of acetone and water was used for separation of the extracts from the spinach samples. The flow rate was set to 1 mL min^{-1} and the temperature to 295 K; the monitoring wavelength was 450 nm. After injection of 20 μL of the spinach extract the column was eluted isocratically with a 84:16 (*v/v*) acetone-water mixture for 21 min. After this a 4-min linear gradient to 97:3 (*v/v*) acetone-water was used. This composition was maintained until the end of the chromatographic run. After the run the initial conditions were re-set within 3 min.

Quantification

Carotenoid stereoisomers were quantified as described elsewhere [8]. The peak areas were determined relative to that of the internal standard, *β-apo*-8′-carotenal, and converted to nanograms by use of standard curves derived from external standards of pure all-*E* lutein, all-*E* zeaxanthin, and all-*E* *β*-carotene. Analytical recovery was determined by adding exact amounts of pure all-*E* lutein and all-*E* zeaxanthin to the spinach samples.

HPLC-NMR

HPLC-NMR experiments were conducted on a Bruker AMX 600 spectrometer equipped with a BPSU-12 (Bruker peak sampling unit) interface (Bruker Analytics, Rheinstetten, Germany) and controlled by HyStar software. The stopped-flow 2D H,H COSY NMR spectrum was recorded at 300 K using an inverse LC-NMR probe with a 120-μL detection cell. Extract (60 μL) from a cooked home-grown spinach (8 weeks growing time) was injected on to the chromatographic column. Water in the mobile phase was replaced by deuterium oxide to reduce the intensity of the solvent signal in the NMR spectrum and to serve as the lock signal for spectrometer stability. To suppress the intense solvent signals from acetone and residual HDO shaped pulses for low-power presaturation (rectangular pulses, length 100 ms) were created for 1.6 s before applying the first 90° pulse. Two hundred and forty increments in the F1 dimension were recorded with 256 transients. The FID were collected into 4 K data points with a spectral width of 13.5 kHz in both dimensions. Data were processed with Bruker 2D WINNMR software, using zero filling with 2 K data points in both dimensions and multiplied by a pure sine wave function before Fourier transformation.

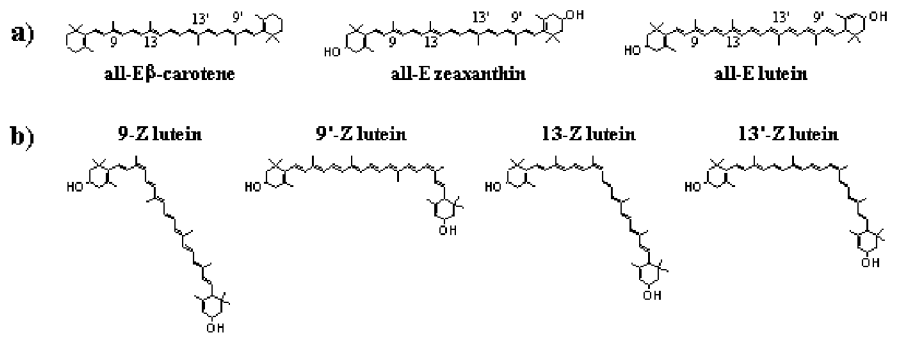

Figure 1. Structures of the carotenoids occurring in spinach: (**a**) all-*E* *β*-carotene, all-*E* zeaxanthin, and all-*E* lutein; (**b**) the main *Z*-stereoisomers of lutein: 9-*Z*, 9′-*Z*, 13-*Z* and 13′-*Z* lutein.

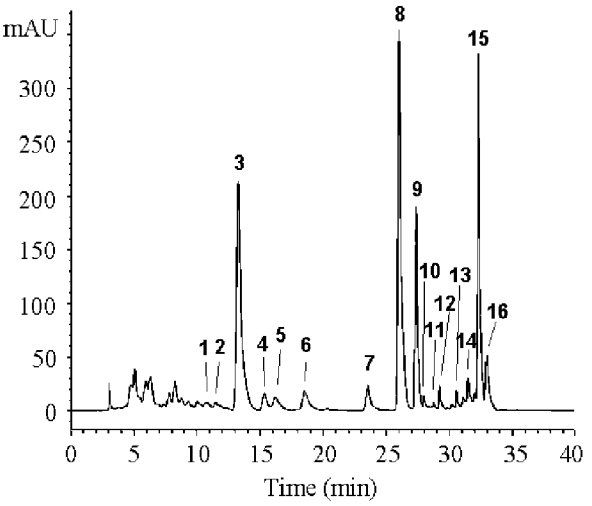

Figure 2. HPLC separation of carotenoid stereoisomers from an extract of cooked, homegrown spinach (eight weeks growing time) on a Bischoff ProntoSIL C_{30} column (3 μm, 200 Å). For peak assignment see Table I. Conditions: flow rate 1 mL min^{-1}, 295 K, UV absorbance detection at 450 nm. Mobile phase acetone-water: 0–21 min 86:14 (*v/v*), 21–25 min linear gradient, 25–40 min 97:03 (*v/v*).

Table I. Peak assignment in Figure 2.

No.	Compound	No.	Compound
1	13-*Z* lutein	9	chlorophyll a
2	13′-*Z* lutein	10	pheophytin b
3	All-*E* lutein	11	pheophytin a
4	All-*E* zeaxanthin	12	13,15-*ZZ* *β*-carotene
5	9-*Z* lutein	13	13-*Z* *β*-carotene
6	9′-*Z* lutein	14	9,13-*ZZ* *β*-carotene
7	Internal standard *β-apo*-8′-carotenal	15	All-*E* *β*-carotene
8	chlorophyll b	16	9-*Z* *β*-carotene

HPLC-APCI-MS

The chromatograph was coupled on-line to an Esquire-LC ion-trap LC-$MS^{(n)}$ system equipped with an APCI interface

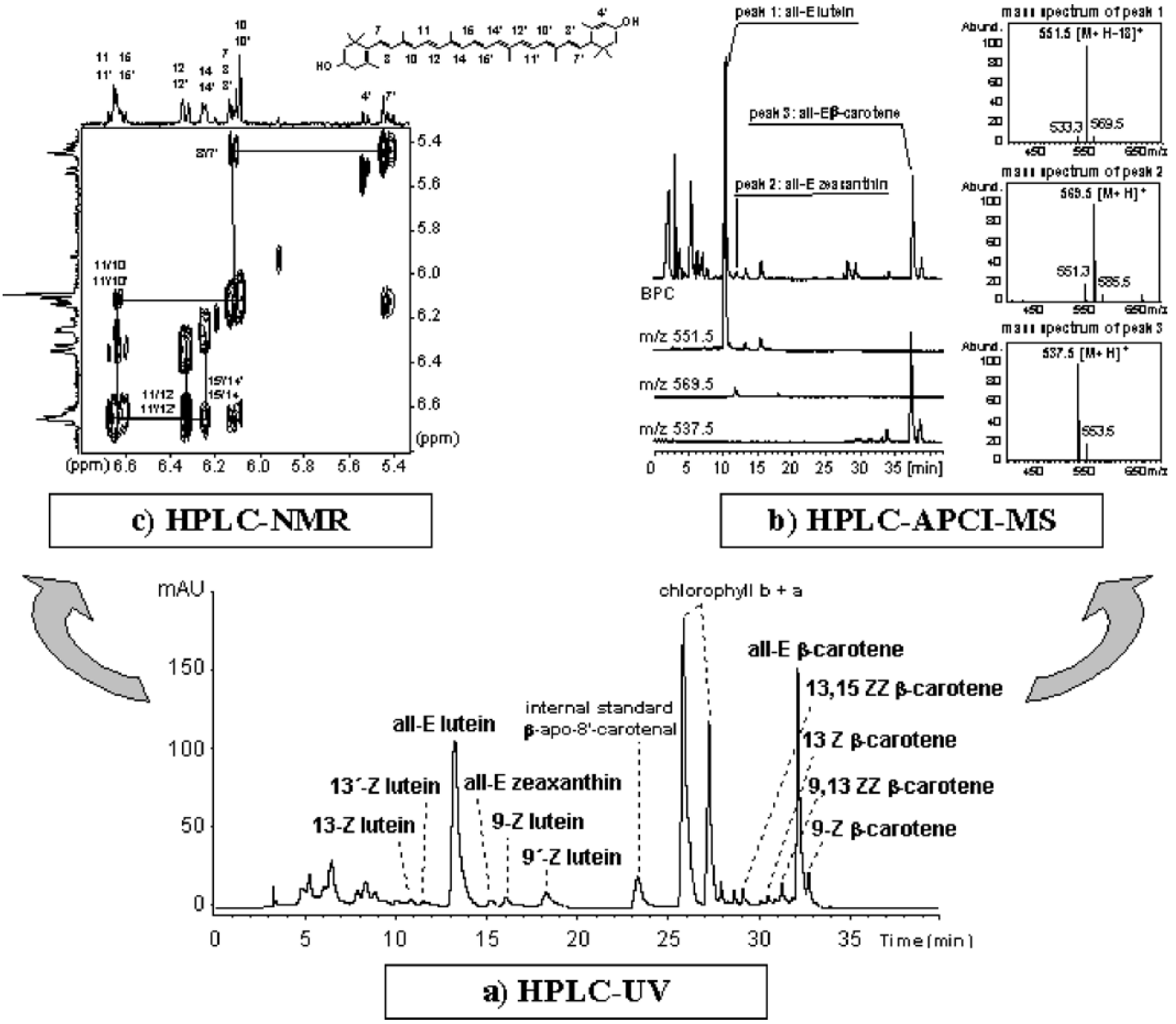

Figure 3. Structural elucidation of carotenoid stereoisomers from spinach by use of hyphenated analytical techniques: (**a**) HPLC-UV chromatogram (450 nm) from cooked commercial spinach. (**b**) Positive-ion HPLC-APCI-MS analysis of a spinach sample. The left side depicts the base peak chromatogram (BPC, upper trace) and the mass traces of *m/z* 551.5, 569.5 and 537.5. The right side shows the mass spectra of the peaks of all-*E* lutein, all-*E* zeaxanthin, and all-*E* β-carotene. (**c**) 2D H,H COSY HPLC-NMR spectrum (600 MHz, olefinic region) of all-*E* lutein obtained from a spinach extract.

(Bruker Daltonics). HPLC-APCI-MS experiments were performed on injection volumes of 10 µL by operating in positive-ion mode with a mass range from *m/z* 200 to 800. The corona discharge voltage was set at 10 kV leading to a current of 8 µA. Nitrogen was used for nebulization of the HPLC mobile phase at a pressure of 15 psig, and as APCI carrier gas, both at 300 °C.

Results and Discussion

Spinach contains both macular pigments, lutein and zeaxanthin, and β-carotene. The structures of these three carotenoids are depicted in Figure 1. All-*E* β-carotene and all-*E* zeaxanthin are both centrosymmetric molecules. Lutein differs from zeaxanthin only in the position of one double bond in one ionene ring system. (Figure 1a). All-*E* lutein is not centrosymmetric, therefore, and the stereoisomers 9-*Z* and 9′-*Z* lutein (or 13-*Z* and 13′-*Z* lutein) are not identical (Figure 1b).

The enhanced shape selectivity of C_{30} stationary phases, suitable for separation of similar carotenoid stereoisomers, is demonstrated in Figure 2 for cooked homegrown spinach (harvested after 8 weeks growth), a major natural source of carotenoids. Because a reversed-phase system was used the more polar xanthophylls lutein and zeaxanthin elute before the nonpolar β-carotene. Quantification is possible because all the main lutein, zeaxanthin, and β-carotene stereoisomers are separated to baseline.

MSPD is the method of choice for extraction of solid biological tissues, because it is a mild and rapid technique, requires little solvent [15], furnishes a highly concentrated extract, and gives quantifiable and reproducible results, because it enables extraction of unstable carotenoid stereoisomers without isomerization and oxidation. The avoidance of sample-preparation artifacts and the completeness of MSPD was proved by recovery tests with pure all-*E* zeaxanthin and all-*E* lutein. A

Table II. Quantitative analysis of the carotenoid *Z*/*E* stereoisomers in a variety of spinach samples.

	Homegrown spinach, harvested after 5 weeks		Homegrown spinach, harvested after 8 weeks		Commercial spinach	
	Raw[a] [mg 100 g^{-1}]	Cooked[a] [mg 100 g^{-1}]	Raw[a] [mg 100 g^{-1}]	Cooked[a] [mg 100 g^{-1}]	Raw[a] [mg 100 g^{-1}]	Cooked[a] [mg 100 g^{-1}]
13-*Z* lutein	0.17 ± 0.01	0.25 ± 0.01	0.21 ± 0.01	0.28 ± 0.02	0.12 ± 0.01	0.13 ± 0.01
13′-*Z* lutein	0.17 ± 0.01	0.31 ± 0.02	0.24 ± 0.01	0.25 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
All-*E* lutein	7.45 ± 0.25	7.59 ± 0.23	9.33 ± 0.30	9.81 ± 0.30	5.71 ± 0.14	4.85 ± 0.19
9-*Z* lutein	0.36 ± 0.02	0.38 ± 0.02	0.71 ± 0.03	0.74 ± 0.03	0.28 ± 0.01	0.22 ± 0.01
9′-*Z* lutein	0.52 ± 0.03	0.63 ± 0.04	1.02 ± 0.04	1.04 ± 0.03	0.20 ± 0.02	0.24 ± 0.01
Total lutein	8.67	9.16	11.51	12.12	6.44	5.71
All-*E* zeaxanthin	0.39 ± 0.02	0.36 ± 0.02	0.63 ± 0.03	0.65 ± 0.02	0.11 ± 0.01	0.13 ± 0.01
13,15-*ZZ* β-carotene	0.14 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.10 ± 0.01
13-*Z* β-carotene	0.31 ± 0.01	0.33 ± 0.02	0.33 ± 0.01	0.37 ± 0.01	0.15 ± 0.01	0.12 ± 0.01
9,13-*ZZ* β-carotene	0.36 ± 0.01	0.31 ± 0.02	0.55 ± 0.02	0.39 ± 0.02	0.48 ± 0.02	0.43 ± 0.03
All-*E* β-carotene	6.15 ± 0.19	6.35 ± 0.19	7.05 ± 0.22	7.40 ± 0.24	3.75 ± 0.10	3.40 ± 0.16
9-*Z* β-carotene	0.92 ± 0.03	1.02 ± 0.04	1.02 ± 0.04	1.12 ± 0.03	0.51 ± 0.03	0.47 ± 0.03
Total β-carotene	7.88	8.17	9.17	9.44	5.01	4.52
Total carotenoids	16.94	17.69	21.31	22.21	11.56	10.36

[a] $n = 5$.

known amount of the pure standards was added to spinach samples before MSPD and recoveries in excess of 98% were obtained for both carotenoids, proving there was no appreciable degradation throughout the extraction step.

Saponification of the spinach extracts is not necessary. The chlorophylls and pheophytins which would be destroyed elute after the xanthophylls and the internal standard and before β-carotene, and do not, therefore, disturb the separation of the carotenoid stereoisomers. A source of errors is also avoided, because carotenoids tend to degrade during saponification [33].

The pathway for structural determination of carotenoid stereoisomers from spinach is shown in Figure 3. By use of HPLC-UV (Figure 3a), the different stereoisomers could be assigned by comparing retention times with those obtained from chromatography of external synthetic standards. Because matrix effects in biological tissues lead to small variations in retention times, and impurities possibly absorb at 450 nm, identification of the carotenoid stereoisomers solely by use of HPLC-UV is not possible. The combined use of HPLC-MS and HPLC-NMR is advantageous, because these coupling techniques are complementary.

HPLC-APCI-MS in positive-ion mode enables assignment of the chromatographic peaks to the different carotenoids within one chromatographic run, as demonstrated in Figure 3b. Stereoisomers of β-carotene (536.5 g mol^{-1}) and zeaxanthin (568.5 g mol^{-1}) can be readily identified by use of the mass traces of *m*/*z* 537.5 of 569.5, respectively, which arise from the protonated molecular ions $[M + H]^+$ (Figure 3b, left). Lutein stereoisomers (568.5 g mol^{-1}) can be distinguished from those of zeaxanthin, although of equal molecular weight, by using the mass trace of *m*/*z* 551.5. Because of the shift of one double bond, elimination of water during the ionization process, leading to a $[M + H - H^2O]^+$ ion, can be observed for lutein. The mass spectra of all-*E* lutein, all-*E* zeaxanthin, and all-*E* β-carotene are shown in Figure 3b (right) and show that positive-ion APCI-MS leads to fragmentation patterns of low complexity.

Because the mass spectra and even the $MS^{(n)}$ spectra of different stereoisomers are identical, HPLC-NMR must be used for structural identification of each stereoisomer. In stopped-flow mode the chromatography is stopped when the peak maximum reaches the NMR detection cell. The acquisition time of an NMR experiment can therefore be adjusted to the concentration of the analyte. Acquisition of 2D NMR spectra is also possible; this gives information about the coupling of each proton and about chemical shift values, δ. The olefinic part of the stopped-flow 2D H,H COSY NMR spectrum of all-*E* lutein obtained from spinach (Figure 3c) shows all the couplings of the olefinic protons. Proton $H_{11/11'}$ at δ = 6.65 ppm has two neighboring protons ($H_{10/10'}$ at δ = 6.11 ppm and $H_{12/12'}$ at δ = 6.33 ppm) and therefore couples with both. It is also apparent that $H_{14/14'}$ couples with $H_{15/15'}$ and $H_{7/7'}$ with $H_{8/8'}$. The NMR spectrum can be easily assigned to lutein, because an additional olefinic signal ($H_{4'}$ at δ = 5.43 ppm) occurs compared with zeaxanthin, because of the shifted double bond [34]. In addition, because proton $H_{7'}$ is in the neighborhood of the aliphatic proton $H_{6'}$ it is shifted to higher field (in contrast with H_7). It must be all-*E* isomer, because most of the quoted and unquoted olefinic protons have identical chemical shifts (except for H_7 and $H_{7'}$).

Results from our quantitative studies on the analysis of carotenoids in a variety of spinach samples by use of MSPD are listed in Table II.

We investigated homegrown spinach harvested, after 5 and 8 weeks, on the day of analysis. We also investigated a commercial spinach product obtained from a local supermarket. All spinach samples were analyzed both raw and processed by cooking in a microwave oven. The results show that all spinach samples contain a large amount of lutein (55%) and β-carotene (43%) isomers and only small amounts of all-*E* zeaxanthin (2%), in accordance with other studies, summarized in the carotenoid database for foods [35].

Homegrown spinach harvested after 8 weeks contains up to twice as much of all carotenoid stereoisomers as the commercial spinach product, as shown in Figure 4. The growing time has a significant effect on the amounts of carotenoids. Increasing the growing time from 5 weeks (Figure 4a) to 8 weeks (Figure 4b) increases the total amount of carotenoids by 25% (from 16.94 to 21.31 mg per 100 g spinach for the raw samples) whereas the distribution of the stereoisomers is almost

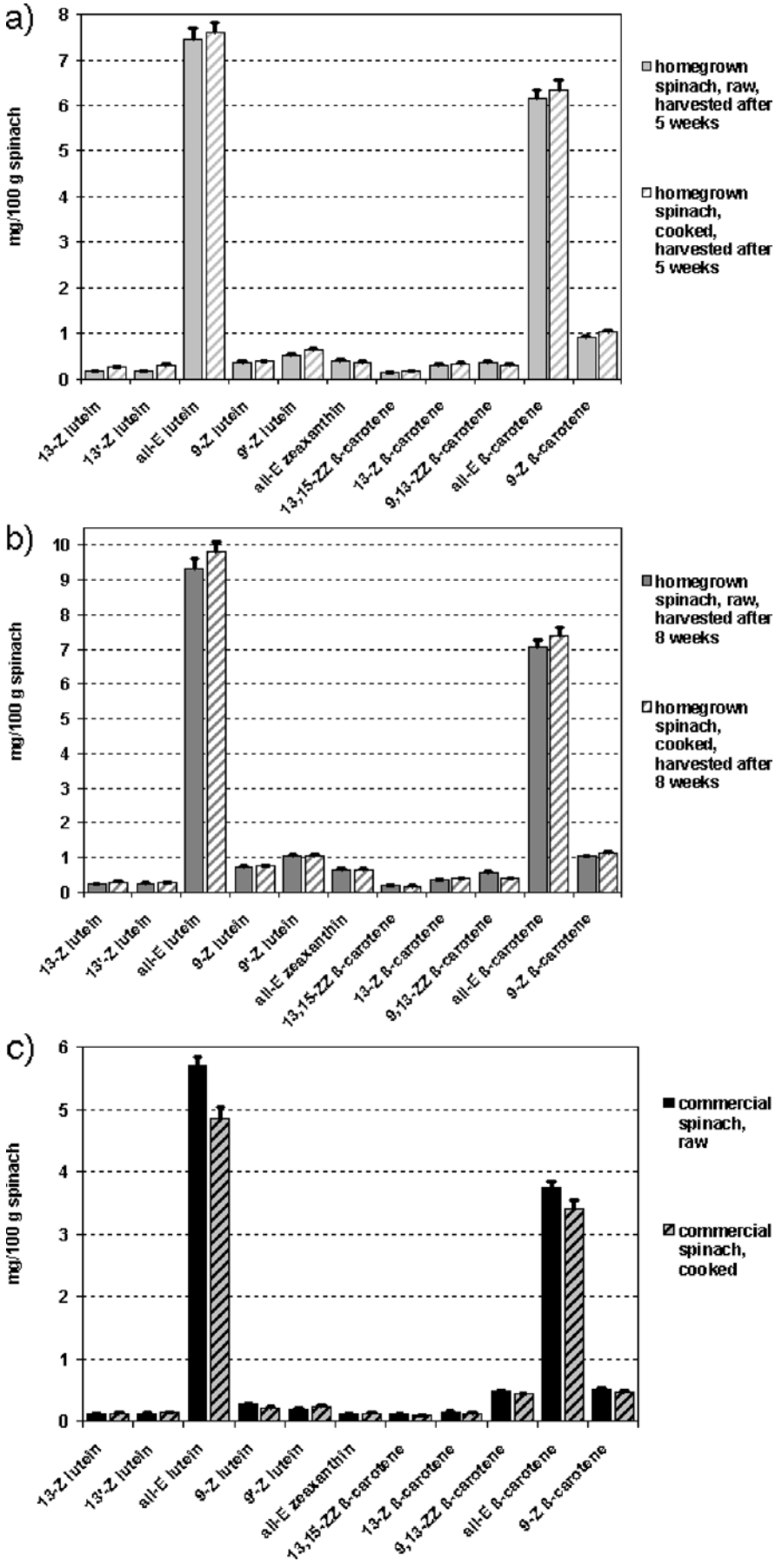

Figure 4. Quantitative analysis of carotenoid stereoisomers in a variety of spinach samples (vertical bars represent the standard deviation of the mean).

the same. Approximately 80% of the total amount of carotenoids in all spinach samples, raw and cooked, are all-*E* stereoisomers.

Microwave cooking has no substantial effect on the amounts of carotenoids in homegrown spinach. The slight increases, 4%, similar for all carotenoid stereoisomers, arise because the carotenoids are tightly bound by the fiber and cells of the vegetable and the cooking process frees them for absorption. Although the raw commercial spinach contains fewer carotenoids (11.56 mg per 100 g spinach), a further decrease of 10%, affecting all stereoisomers similarly, occurred as a result of the cooking process (Figure 4c). The carotenoids in this statement are destroyed by the pretreatment during production.

Conclusion

On the basis of MSPD in combination with HPLC-UV, HPLC-APCI-MS, and HPLC-NMR we have established a method for artifact-free quantitative determination of carotenoid stereoisomers in biological tissues. Homegrown spinach was found to contain significantly more lutein, zeaxanthin, and β-carotene stereoisomers than commercial spinach.

MSPD enables rapid, mild, complete, and reproducible extraction and enrichment of these phytonutrients from spinach samples, and is suitable for quantification. That isomerization and oxidation of the light- and air-sensitive carotenoid stereoisomers do not occur can be proved by recovery tests with pure all-*E* zeaxanthin. Positive-ion HPLC-APCI-MS using C_{30} stationary phases enables assignment of the chromatographic peaks to the different carotenoids whereas online HPLC-NMR coupling enables unambiguous elucidation of the structures of carotenoid stereoisomers.

We identified the same carotenoid stereoisomers in spinach samples as are present in the human macula (lutein and zeaxanthin stereoisomers) or serum (β-carotene). Spinach as a natural source of carotenoids contains a large amount of lutein and β-carotene and only a small amount of zeaxanthin. Homegrown spinach contains twice as much of all carotenoid stereoisomers as commercial spinach. Cooking the spinach had no negative effect for homegrown spinach but the amount in commercial spinach was

further reduced. Homegrown spinach might therefore be more suitable for nutritional supplementation studies.

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