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Isolation of carotenoids from plant materials and dietary supplements by high-speed counter-current chromatography

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Abstract

Methods for the isolation of lipophilic pigments from crude extracts of plant materials (spinach and sweet corn) by high-speed counter-current chromatography (HSCCC) were developed. Particular attention was given to (*all-E*)-lutein and (*all-E*)-zeaxanthin. However, the concomitant pigments neoxanthin, violaxanthin and β-carotene as well as chlorophylls *a* and *b* were also considered. Furthermore, for the first time dietary supplements containing lutein and zeaxanthin were also used as a source for the recovery of carotenoids. Due to their simple matrix (oily excipient in soft gelatine capsules), sample preparation was facilitated and consumption of solvents was minimized. The carotenoids were characterized by ^{1}H NMR spectroscopy, by LC/APcI-MS in the positive ionization mode, and by UV–vis spectroscopy. Data showed that the target compounds were of high purity (90–93%). Lutein and zeaxanthin may be used as reference substances for analytical purposes.

Keywords: HSCCC; High-speed counter-current chromatography; Lutein; Zeaxanthin; Violaxanthin; Neoxanthin; β-Carotene; Chlorophyll *a*; Chlorophyll *b*

1. Introduction

Carotenoids represent one of the most widespread and important group of natural pigments. They are commonly present as plant pigments, but some also occur in microorganisms and animals. Apart from their function as colour, carotenoids containing a β-ionone ring act as precursors of vitamin A and are reported to be excellent scavengers of singlet oxygen and other reactive oxygen species [1]. Since carotenoids are not synthesized by humans, their ingestion with the diet is a prerequisite. The major carotenoids in human plasma are β-carotene, lycopene, β-cryptoxanthin, lutein, α-carotene and zeaxanthin [2]. Numerous epidemiological studies suggest an inverse correlation of carotenoid intake and the incidence of degenerative diseases [3–6]. Especially lutein and zeaxanthin have been associated with a protective activity against the two common eye diseases of ageing, cataract and age-related macular degeneration [7]. Consequently, there is an increasing interest in dietary supplements and functional food providing larger amounts of carotenoids. It is expected that in 2010 this market will have grown at $ 500 billion [8]. As a result, methods for the quantification of carotenoids and other bioactive constituents present in these types of food are urgently needed. However, the evident lack of reference compounds is a major concern. Furthermore, our recent investigations have shown that even commercially available standard substances are not sufficiently specified [9].

HSCCC is widely used in the preparative separation of natural products. Besides a much larger separation capacity compared to HPLC, HSCCC allows the direct application of crude extracts and an excellent recovery of the analytes. Because of their lipophilic nature methods for the separation of carotenoids are rare [10–15], or only suitable for the recovery of water-soluble carotenoids [16,17]. Therefore, the objective of the present study was to develop methods for the isolation of carotenoids to be used as reference compounds for ana-

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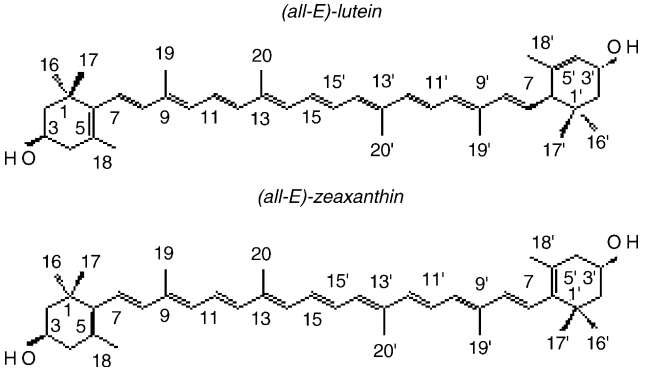

Fig. 1. Structures of (*all-E*)-lutein and (*all-E*)-zeaxanthin.

lytical purposes. Owing to their relevance as constituents of dietary supplements, particular attention was given to (*all-E*)-lutein and (*all-E*)-zeaxanthin (Fig. 1). Because the xanthophylls were isolated from spinach and sweet corn, the concomitant pigments violaxanthin, neoxanthin, β-carotene and chlorophylls *a* and *b* of spinach were also included in the study. Furthermore, the utilization of dietary supplements as source for carotenoids is reported for the first time. For environmental and health protection, the use of halogenated hydrocarbons was avoided.

2. Experimental

2.1. Materials

All chemicals used were purchased from VWR (Darmstadt, Germany) and were of reagent grade. HPLC solvents were of gradient grade. For HPLC analyses and sample preparations ultrapure water was employed. (*All-E*)-lutein and (*all-E*)-zeaxanthin were supplied by Hoffmann-La Roche (Basel, Switzerland). Spinach (*Spinacia oleracea* L.) and sweet corn (*Zea mays* L.) were purchased from the local market. Dietary supplements consisting of soft gelatin capsules containing mixtures of carotenoids were purchased in a local drugstore and from an internet shop.

2.2. Sample preparation

2.2.1. Preparation of crude plant extracts

To avoid degradation and isomerization of carotenoids, amber glass ware was used and extraction was performed under dim light. Fresh spinach (approx. 2600 g) was washed and cut into small pieces. Kernels of sweet corn (approx. 3900 g) were removed from the cob with a knife and homogenized with a cutter. After lyophilisation of the minced plant material, the samples were extracted in a blender using hexane/acetone (1:1, v/v) containing butylated hydroxytoluene (BHT) (100 mg/l) and butylated hydroxyanisole (BHA) (100 mg/l) as antioxidants. The solutions were filtered (cellulose, white ribbon grade, Schleicher & Schuell, Dassel, Germany) to remove particles. Organic solvents were evaporated in vacuo ($T < 30\,^{\circ}C$). The residue was dissolved in 300 ml of hexane and washed three times with 900 ml of water to remove water-soluble compounds (e.g. sugars, starch and protein). Organic solvents were evaporated in vacuo ($T < 30\,^{\circ}C$), and the dried residues were stored under nitrogen at $-80\,^{\circ}C$. Portions thereof were used for HSCCC.

2.2.2. Preparation of crude extracts of dietary supplements

An appropriate number of capsules, depending on the carotenoid content, was cut with a scalpel and extracted with 50 ml of hexane. The organic layer was evaporated in vacuo ($T < 30\,^{\circ}C$), and the residue was used for HSCCC. Due to their particles content, the organic extract of the lutein containing dietary supplement was transferred to an amber glass separatory funnel and washed twice with 50 ml of water. Further steps of sample preparation were carried out as described above.

2.3. HSCCC

2.3.1. Apparatus

HSCCC was performed using a model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). The separating device consisted of three preparative coils connected in series. The PTFE tubes had an inner diameter of 0.8 mm and a total volume of 325 ml. Considering a distance of $r = 3.8$–5.7 cm from the coil to the holder shaft and of $R = 7.6$ cm from the column axis to the central axis of the centrifuge, the β-value ($\beta = r/R$) varied from 0.50 (internal terminal) to 0.75 (external terminal). The rotation speed was adjustable, ranging from 0 to 2000 rpm. The HSCCC system was equipped with a solvent delivery module BT 8100 (Biotronic, Maintal, Germany), a UV–vis detector BT 8200 (Biotronic, Maintal, Germany), a fraction collector 2211 Superrac (LKB, Bromma, Sweden), an integrator D-2500 (Merck Hitachi, Darmstadt, Germany), and an injection valve with a 4 ml sample loop.

2.3.2. Selection of the solvent system

Without solid support, solute retention solely depends on the partition coefficient *K* representing the ratio of solute distributed between a mutually equilibrated biphasic system. Its determination was performed by adding a small amount of carotenoid standard to both equilibrated solvent phases. The sample was vigorously shaken with the two phase system and the absorbances of upper and lower phase were determined spectrophotometrically. The *K* value was calculated according to the equation $K = c_{\text{upper phase}}/c_{\text{lower phase}}$.

2.3.3. Preparation of the biphasic solvent system and sample solution

For the isolation of lutein and zeaxanthin, a biphasic solvent system (hexane/ethanol/water, 6:5:1.3, v/v) was applied.

The solvent mixture was thoroughly equilibrated in a separatory funnel by vigorously shaking for 2 min at ambient temperature. The phases were separated immediately before use. Sample solutions were prepared by dissolving the crude extract in a mixture of upper and lower phase (1:1, v/v).

2.3.4. Isolation of pigments by HSCCC

The HSCCC system was operated in the reversed-phase mode. After loading the column with the stationary phase (upper phase), the counter-current partition was performed at a revolution speed of 1050 rpm, while the mobile phase (lower phase) was pumped into the head end of the column at a flow rate of 1.0 ml/min. For accelerated isolation of the pigments, the dissolved samples were immediately injected into the column without establishing the hydrodynamic equilibrium. The pressure of the system was approximately 70 p.s.i. Chromatographic runs were monitored at 445 nm for lutein and at 450 nm for zeaxanthin. Fractions were collected in intervals of 3 min.

2.4. Analytical chromatography

The HPLC system consisted of a model 2690 Waters separation module equipped with an autosampler injector, a model Jetstream 2 plus Waters column oven and a model 2996 Waters UV–vis photodiode array detector, controlled by Millennium 32 (Version 3.20) workstation (Waters, Milford, MA, USA). Chromatographic analyses were performed using a 3 μm analytical C_{30} reversed phase column (150 mm × 3.0 mm I.D.) protected by a 3 μm C_{30} reversed phase guard column (10 mm × 4.0 mm I.D.) (YMC, Wilmington, MA, USA) at 20 °C and a flow rate of 0.42 ml/min.

For the separation of xanthophyll isomers and chlorophylls, eluent A consisted of methanol/*tert*-butyl methyl ether (MTBE)/water (92:4:4, v/v), and eluent B of MTBE/methanol/water (90:6:4, v/v). A linear gradient from 100% A to 15% B within 55 min was applied.

2.5. NMR spectroscopy

The ^{1}H NMR spectra of the isolated compounds were recorded on a Varian Unity Inova 500 spectrometer with $CDCl_3$ as the solvent ($\delta H = 7.27$ ppm).

2.6. Mass spectrometry

LC–MS analyses were performed on an HPLC series 1100 (Agilent, Waldbronn, Germany) coupled with a mass spectrometer Esquire 3000 + ion trap fitted with an APcI source (Bruker, Bremen, Germany). The HPLC system was equipped with ChemStation software, a degasser G1379A, a binary gradient pump G1312A, an autosampler G1313A, a column oven G1316A, and a diode array detector G1315B. Positive ion mass spectra were recorded in the range *m*/*z* 50–1000. Nitrogen was used as dry gas at a flow rate of 4.5 l/min and at a pressure of 50 p.s.i. Temperatures were set at 350 and 400 °C for the nebulizer and the vaporizer, respectively. Corona was set at 4000 nA. The chromatographic conditions of the analytical separation were applied.

2.7. Quantification of carotenoids

Quantification was performed on a UV–vis spectrometer Lambda 20 (Perkin-Elmer, Nowalk, CT, USA) and based on UV–vis spectral analysis using specific absorption coefficients ($A^{1\%}_{1cm}$ for (*all-E*)-lutein: 2550 at 445 nm (ethanol) and (*all-E*)-zeaxanthin: 2540 at 450 nm (ethanol) [18,19].

3. Results and discussion

3.1. HSCCC solvent system

Since HSCCC has no solid support, retention of the stationary phase in the column is a prerequisite for high peak resolution. Ideally, the mobile phase passes the system, while more than 50% of the stationary phase is retained. The retention volume is highly correlated with the separation time of the two phases in a test tube which should not exceed 20 s after vigorous shaking. Furthermore, partition coefficients *K* ranging from $0.5 \leq K \leq 1$ are required for an efficient resolution and short elution time [20]. In the present study, retention of the stationary phase was in the range of 70–85%. The retention of the stationary phase relative to the total column volume was determined from the volume of the stationary phase collected during the separation. Using the stationary and mobile phases described in Section 2.3.3, partition coefficients *K* and separation times were 0.77 and 13 s for lutein, and 0.60 and 13 s for zeaxanthin, respectively.

3.2. Lutein

3.2.1. Isolation of lutein from spinach

In preliminary investigations the predominant pigments of a crude spinach extract were characterized by analytical HPLC (Fig. 2A). Besides lutein, violaxanthin, neoxanthin, β-carotene (not shown as peak eluted after 55 min), as well as chlorophylls *a* and *b* were detected. The separation of pigments obtained from 200 mg of crude spinach extract by HSCCC is shown in Fig. 3. The carotenoids were eluted in the order of decreasing polarity (neoxanthin > violaxanthin > lutein > β-carotene). Interestingly, a reversed elution order of neoxanthin and violaxanthin as well as of zeaxanthin and lutein was observed in HPLC separation using a C_{30} stationary phase. This phenomenon might be explained by the shape selectivity of these phases, which are capable of discriminating structurally related compounds. Compared to a previous study using microalgae for the recovery of lutein [10], isolation from spinach is considered a more easily available source. Furthermore, saponification of lipids is not required and the use of halogenated hydrocarbons [11] may be avoided. Another problem with microalgae con-

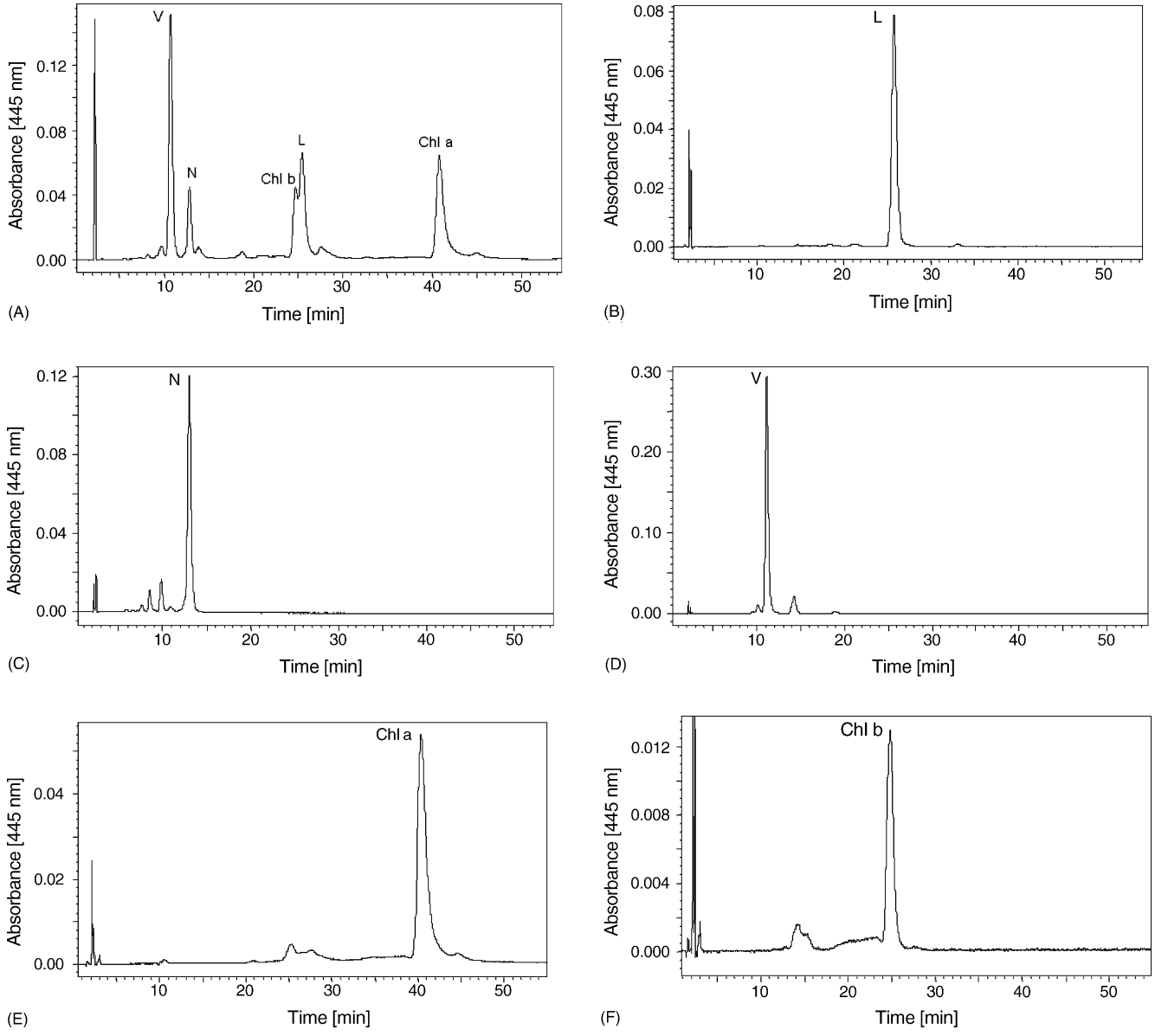

Fig. 2. HPLC separations of pigments extracted from spinach and several HSCCC fractions. (V) Violaxanthin, (N) neoxanthin, (Chl b) chlorophyll *b*, (L) lutein and (Chl a) chlorophyll *a*. (A) The crude sample (B) HSCCC fraction of peak L (C) HSCCC fraction of peak N (D) HSCCC fraction of peak V (E) HSCCC fraction of peak Chl a (F) HSCCC fraction of peak Chl b. Conditions: column: C_{30} column (150 mm × 3.0 mm I.D., 3 μm) and a guard column (10 mm × 4.0 mm I.D., 3 μm); eluent A methanol/*tert*-butyl methyl ether/water (92:4:4, v/v); eluent B *tert*-butyl methyl ether/methanol/water (90:6:4, v/v) in gradient mode; flow-rate: 0.42 ml/min.

taining almost equal amounts of (*Z*)- and (*E*)-isomers, which would require further separation of the stereoisomers, may be circumvented.

The area of the lutein peak comprised 93% of the total peak area as determined by HPLC at 445 nm (Fig. 2B). Concomitant small side peaks represent (*Z*)-isomers of (*all-E*)-lutein. NMR investigations of the carotenoid containing fraction revealed that apart from lutein traces of other lipophilic compounds such as triglycerides were present. Because the ^{1}H NMR spectrum was not affected by overlapping signals of lipophilic impurities and the olefinic protons of the carotenoid at $\delta = 6$–7 ppm, the isomeric purity of lutein could be determined. In plants, carotenoids are predominantly present in their (*all-E*)-configuration, although the highly unsaturated polyene chain of carotenoids is prone to isomerization caused by several factors such as heat, light and acids [18], (*Z*)-stereoisomers were detected in small amounts, only. In a previous work 21% lutein (*Z*)-isomers were found in fresh spinach [21]. By comparison of the ^{1}H NMR spectrum with literature data (*all-E*)-lutein was unambiguously confirmed as the predominant isomer with a purity >95%. Because the lipophilc impurities detected by NMR spectroscopy did not show any absorption in the visible region, spectrophotometric quantification of lutein at 445 nm could be accomplished.

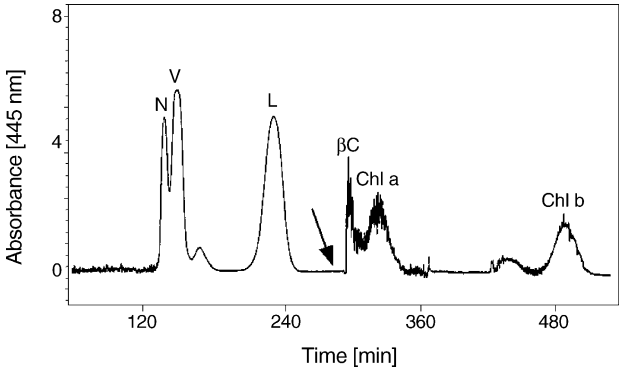

Fig. 3. HSCCC separation of pigments extracted from spinach. (N) Neoxanthin, (V) violaxanthin, (L) lutein, (βC) β-carotene, (Chl a) chlorophyll *a* and (Chl b) chlorophyll *b*. The arrow indicates the stop of rotation (295 min) to elute the less polar compounds in reversed mode (tail to head). Conditions: solvent system: hexane/ethanol/water (6:5:1.3, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.0 ml/min; revolution speed: 1050 rpm; sample size: 200 mg.

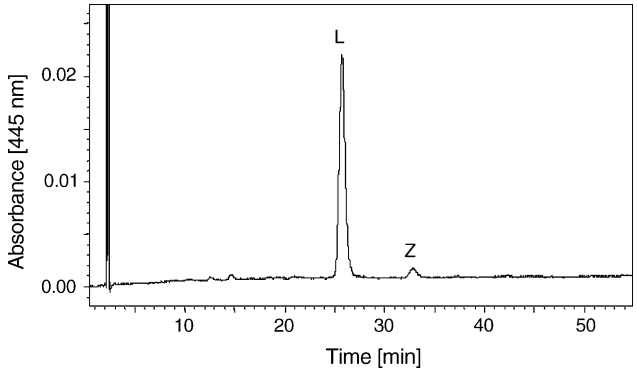

Fig. 4. HPLC separation of xanthophylls extracted from a dietary supplement. (L) Lutein and (Z) zeaxanthin. Conditions: column: C_{30} column (150 mm × 3.0 mm I.D., 3 μm) and a guard column (10 mm × 4.0 mm I.D., 3 μm); eluent A methanol/*tert*-butyl methyl ether/water (92:4:4, v/v); eluent B *tert*-butyl methyl ether/methanol/water (90:6:4, v/v) in gradient mode; flow-rate: 0.42 ml/min.

Each chromatographic run yielded 0.8 mg (*all-E*)-lutein being sufficient for calibration purposes. If larger amounts are required, the portion of crude extract applied to HSCCC may be rised up to 600 mg. The structure of (*all-E*)-lutein was confirmed by UV–vis spectra, mass spectra and ^{1}H NMR spectra. Spectroscopic data (λ_{max}) of (*all-E*)-lutein determined by photodiode-array detection in the corresponding HPLC solvents (445 and 473 nm), mass spectra and ^{1}H NMR spectra were in agreement with published data [19,21,22].

Although particular attention was given to the recovery of lutein, further accessory pigments of spinach were also included in the study. As shown in Fig. 3, the separation of neoxanthin (N) and violaxanthin (V) was sufficient for the simultaneous isolation of both compounds from the spinach extract. To improve the resolution, modification of the solvent system appears to be successful. HPLC chromatograms of the two isolated carotenoids are shown in Fig. 2C and D. Spectroscopic data (λ_{max}) of neoxanthin and violaxanthin as determined by photodiode-array detection in the corresponding HPLC solvents were 439 and 468 nm, and 435 and 464 nm, respectively. Mass spectrometric investigation revealed a $[M+H]^+$ ion of *m*/*z* 601.4 for violaxanthin and a base peak at *m*/*z* 583.8 for neoxanthin, corresponding to $[M+H-H_2O]^+$. Mass spectra and UV–vis spectra were in agreement with published data [19,23,24].

Due to their higher affinity to the stationary phase, the less polar pigments of the spinach extract were still retained in the HSCCC. Therefore, to complete pigment isolation, the flow and the rotation of the HSCCC were stopped and the elution mode was reversed (tail to head) after the elution of the lutein peak. After setting the flow rate at 0.8 ml/min, β-carotene, chlorophylls *a* and *b* were recovered (Fig. 3), and the fractions were investigated by analytical HPLC (Fig. 2E and F). UV–vis spectra (λ_{max}) determined in the corresponding HPLC solvents and mass spectra were as follows: β-carotene, 451 and 478 nm, *m*/*z* = 537.2; chlorophyll *a*, 432 and 665 nm, *m*/*z* = 893.5; and chlorophyll *b*, 469 and 650 nm, *m*/*z* = 907.4 being in agreement with the literature [25]. To the best of our knowledge this paper marks the first report on the isolation of chlorophylls by HSCCC.

3.2.2. Isolation of lutein from a dietary supplement

Compared to crude plant extracts, dietary supplements represent a less complex matrix and are therefore a more convenient source for the recovery of bioactive compounds. Carotenoids used as supplements are either synthetic or extracted from plant materials, dissolved in an excipient (vegetable oil), and encapsulated in soft gelatin [26]. Since the capsule content can directly be used for HSCCC after dissolution, only small amounts of organic solvents are required for sample preparation. As shown by analytical HPLC, the lutein containing supplement extract contained minor amounts of zeaxanthin (Fig. 4). The HSCCC separation of lutein and zeaxanthin is depicted in Fig. 5. The lutein fraction recovered from the eluate was characterized by UV–vis and NMR spectroscopy. As determined by HPLC at 445 nm the area of

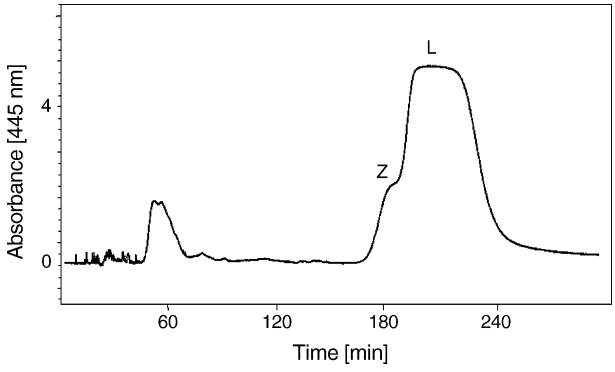

Fig. 5. HSCCC separation of xanthophylls extracted from a dietary supplement. (L) Lutein and (Z) zeaxanthin. Conditions: solvent system: hexane/ethanol/water (6:5:1.3, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.0 ml/min; revolution speed: 1050 rpm; sample size: content of three capsules.

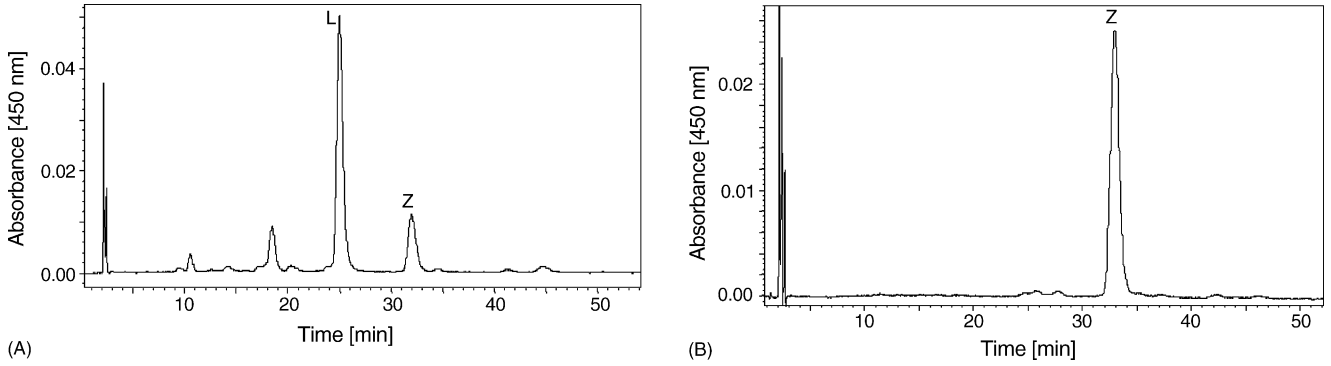

Fig. 6. HPLC separations of xanthophylls from sweet corn extract and of the target compound. (Z) Zeaxanthin and (L) lutein. (A) The crude sample (B) the purified HSCCC fraction of peak Z + L. Conditions: column: C_{30} column (150 mm × 3.0 mm I.D., 3 μm) and a guard column (10 mm × 4.0 mm I.D., 3 μm); eluent A methanol/*tert*-butyl methyl ether/water (92:4:4, v/v); eluent B *tert*-butyl methyl ether/methanol/water (90:6:4, v/v) in gradient mode; flow-rate: 0.42 ml/min.

the lutein peak amounted to >93% of the total peak area. The lutein yield, determined by spectrophotometry, was 5.1 mg. Considering the contents of three capsules containing 2 mg of lutein each (according to the product specification) used for the isolation, a yield of 85% was achieved. NMR spectrocopic investigations revealed that lipophilic impurities were also present, however, not affecting the quantification. (*Z*)-Stereoisomers were detected in trace amounts only (<4%).

3.3. Zeaxanthin

3.3.1. Isolation of zeaxanthin from sweet corn

Contrary to lutein, zeaxanthin usually represents a minor constituent of most plants. As can be seen from the HPLC separation of lutein and zeaxanthin from a crude extract, even in sweet corn lutein is the predominant xanthophyll (Fig. 6A). The HSCCC chromatogram of the separation of xanthophylls is shown in Fig. 7. Since preliminary investigations demonstrated that a single HSCCC run did not provide sufficient amounts of zeaxanthin, portions of 3 ml of the crude extract were injected seven times and the combined lutein and zeaxanthin fractions were pooled. Due to the quantitative predominance of lutein, a satisfactory separation of the xanthophylls solely by HSCCC could not be accomplished. After HSCCC a semi-purified extract was obtained which contained >97% zeaxanthin and lutein (ratio 1:4). Therefore, further clean-up by preparative HPLC according to a recently described method [21] was indispensable, finally yielding 0.8 mg (*all-E*)-zeaxanthin. The area of the zeaxanthin peak amounted to 93% of the total peak area as determined by HPLC at 450 nm (Fig. 6B). Small peaks next to (*all-E*)-zeaxanthin represent (*Z*)-isomers of zeaxanthin.

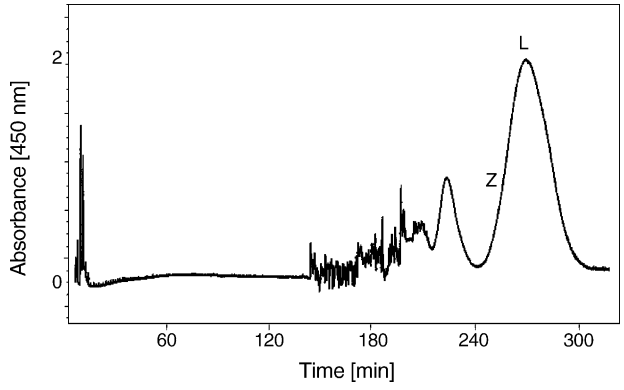

Fig. 7. HSCCC separation of xanthophylls extracted from sweet corn. (Z) Zeaxanthin and (L) lutein. Conditions: solvent system: hexane/ethanol/water (6:5:1.3, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.0 ml/min; revolution speed: 1050 rpm; sample size: 3 ml.

Spectroscopic data (λ_{max}) of (*all-E*)-zeaxanthin determined by photodiode-array detection in the corresponding HPLC solvents (450 and 477 nm), ^{1}H NMR and mass spectra were in agreement with previously published studies [19,21,22]. According to NMR spectroscopic investigations, the proportion of (*all-E*)-zeaxanthin was >95%, while only negligible amounts of (*Z*)-isomers were present. From these data it becomes evident that the amount of zeaxanthin (*Z*)-isomers (7%) detectable in fresh sweet corn [21] was not increased during HSCCC separation. Further non-interferring lipophilic compounds originating from the oily excipient were detected. To our knowledge this marks the first report on the isolation of zeaxanthin by HSCCC.

3.3.2. Isolation of zeaxanthin from a dietary supplement

According to the labelled specification, each capsule used for preparative isolation contained 5 mg of zeaxanthin and 10 mg of lutein. The predominance of lutein resulted in a poor resolution of zeaxanthin and lutein (Fig. 8A). For zeaxanthin enrichment, the column eluate was collected from 85 to 140 min. Because HPLC analysis revealed that the combined partially purified fractions still contained 13% lutein, they were again subjected to HSCCC. From Fig. 8B it can be seen that the ratio of zeaxanthin to lutein was considerably increased after re-chromatography. A total of 0.9 mg (*all-E*)-zeaxanthin with a purity of >90% was recovered. As already described above, traces of lipophilic compounds originating from the oily excipient did not affect the quantification.

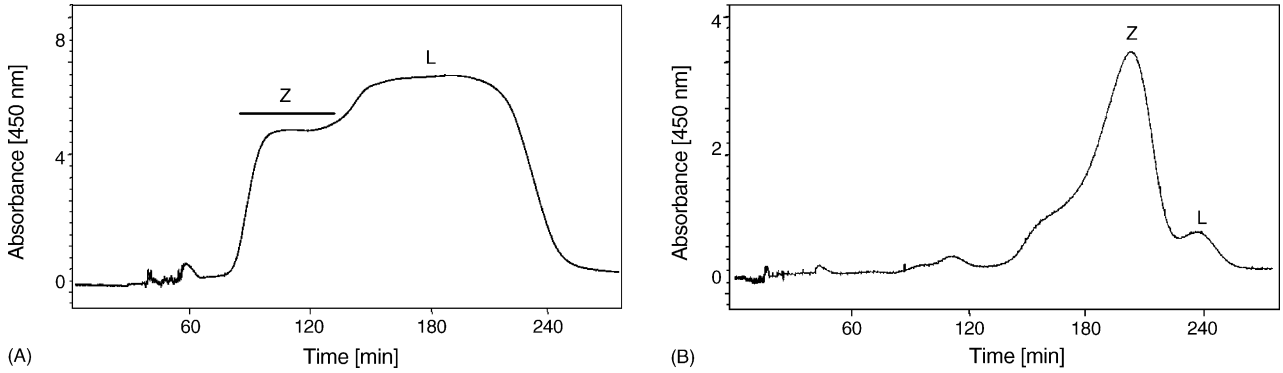

Fig. 8. HSCCC separations of xanthophylls extracted from a dietary supplement. (Z) Zeaxanthin and (L) lutein. (A) The crude sample; sample size: content of one capsule (B) the HSCCC fraction of peak Z after repeated counter-current chromatography. Conditions: solvent system: hexane/ethanol/water (6:5:1.3, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.0 ml/min; revolution speed: 1050 rpm.

4. Conclusions

The results obtained in the present study demonstrate that HSCCC is a powerful technique for the isolation of bioactive compounds from plant materials, as exemplified for the xanthophylls lutein and zeaxanthin from spinach and sweet corn extracts. Despite their structural similarity, differing only in the position of one double bond in the ionone ring, the resolution capacity of HSCCC was sufficient for the isolation of both xanthophylls without using halogenated solvents. Except for zeaxanthin recovered from sweet corn extracts, the compounds obtained may be used as references substances without additional clean-up. If a higher purity is desired, removal of lipids by saponification or purification by preparative HPLC would be helpful. From our study it becomes also evident that the use of dietary supplements as a source of carotenoids is a promising approach, which has so far not been considered. Due to the comparatively simple matrix, less time for sample preparation and only small amounts of solvents are required. A similar approach may also be suitable for the isolation of biologically active compounds other than carotenoids, e.g. phytosterols and polyphenolics.

Acknowledgments

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