Development and Evaluation of a New Method for the Determination of the Carotenoid Content in Selected Vegetables by HPLC and HPLC–MS–MS

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Abstract

Epidemologic studies have shown inverse correlation between the consumption of carotenoid-rich vegetables and the incidence of cancer. Therefore, analytical techniques for the quantitative determination of carotenoids in complex sample matrices are important. The most used method is reversed-phase (RP)-high-performance liquid chromatography (HPLC). In this study, seventeen mobile-phase systems described in the literature and six RP-HPLC columns with differences in particle size and porosity are evaluated. Derived from these results, a new mobile-phase (acetonitrile, methanol, chloroform, and *n*-heptane) including solvent modifiers is presented, which allows an improved and more efficient separation of carotenoids. From all columns tested, the best chromatographic parameters are found using a silica C_{18} column (250 × 2 mm, 5 µm, 100 Å). As was found, absorbance detection at 450 nm allows the determination of the carotenoids down to the picogram range with good linearity ($R^2 > 0.98$). For the identification and quantitation of carotenoids in complex sample matrices (containing additionally other ultraviolet-absorbing compounds), the optimized RP chromatographic system is coupled to a mass spectrometer (MS) using an atmospheric pressure ionization interface. The calibration plots show high linearity ($R^2 > 0.99$), and the detection limit is found in the lower nanogram range. Furthermore, collision-induced dissociation in the ion source allows for the identification of carotenoids by their characteristic fragmentation pathways. In this study, a total of nine species of vegetables commonly consumed in Central Europe are analyzed for their contents of carotenoids (namely lutein, zeaxanthin, β-cryptoxanthin, and β-carotene) by RP-HPLC and RP-HPLC–MS–MS. It is found that good sources for lutein are spinach, kale, and broccoli, and sources for β-carotene are broccoli, spinach, kale, carrots, and tomatoes. This new method is an improvement for the identification and quantitation of carotenoids in complex biological tissues.

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Introduction

The name "carotene" is derived from carrot root (*Daucus carota*), which was isolated as a colored pigment in 1831. Chemically, carotenoids are conjugated hydrocarbons that may be further classified as carotenes (without any oxygen molecules) and xanthophylls (with one or more oxygen molecules). Carotenoids are widespread natural pigments found in all kingdoms of the living world. They are recognizable from the bright colors (yellow, orange, red, or purple) that they often confer on plant and animal organs. More than 600 natural carotenoids have been identified, and the number is still increasing (1). Carotenoids are biosynthesized by bacteria, algae, fungi, and plants (2), but not by animals, which have to obtain them from their food. They play an essential biological role in light-energy collection and photoprotection (3). A large number of epidemologic studies have shown that fruit and vegetable consumption is associated with a reduced risk for many cancers and other chronic diseases (4,5). Therefore, scientists have become interested in the five carotenoids with the highest known blood concentration in human beings: α-carotene, β-carotene, lycopene, lutein, and β-cryptoxanthin (6–8). Three of these carotenoids (α-carotene, β-carotene, and β-cryptoxanthin) also have provitamin A activity and can be converted in the body to all-trans retinol.

Traditional separation methods for carotenoids employ open-column and thin-layer chromatography (9). The development of high-performance liquid chromatographic (HPLC) methods has contributed recently to advances in the analysis of carotenoids. HPLC methods use isocratic and gradient mobile phases in either reversed-phase or normal-phase mode (10–14). In spite of the fact that carotenoids are easily oxidized, it is useful to add antioxidans such as butylated hydroxytoluene to the mobile phase and keep the column temperature constant and low (15,16). Coupling a photodiode array detector to the HPLC allows for a continuous collection of spectrophotometric data during the analysis (17), which in some cases is not

sufficient for identification. However, the complexity of the mixtures in which these carotenoids are found is quite high. For this reason, much effort has been devoted to developing HPLC–mass spectrometry (MS)–MS methods with mainly atmospheric pressure ionization interfaces (APCI) or electrospray ionization interfaces (ESI) (18–20). Furthermore, MS detection offers the possibility to use collisionally induced dissociation (CID) in order to obtain fragment ions of structural relevance, which can be used as an additional "fingerprint" for identifying carotenoids in these highly complex mixtures (21). In addition, the elucidation of structures and stereochemistry has been made possible using HPLC–nuclear magnetic resonance coupling (22,23). In this work, we present the development and evaluation of a new method with improved recoveries and selectivities for the HPLC and HPLC–MS–MS determination of lutein, zeaxanthin, β-cryptoxanthin, and β-carotene in selected vegetables.

Table I. Solvents and Extinction Coefficients for the Preparation of Working Solutions

Carotenoid	Solvent	λ	$E^{1\%}_{1cm}$	E^{mM}_{1cm}	MWt
Lutein	ethanol	445	2550	145.1	569
Zeaxanthin	*n*-hexane	451	2480	141.1	569
β-Cryptoxanthin	*n*-hexane	451	2460	136.0	553
β-Carotene	*n*-hexane	450	2560	137.4	537

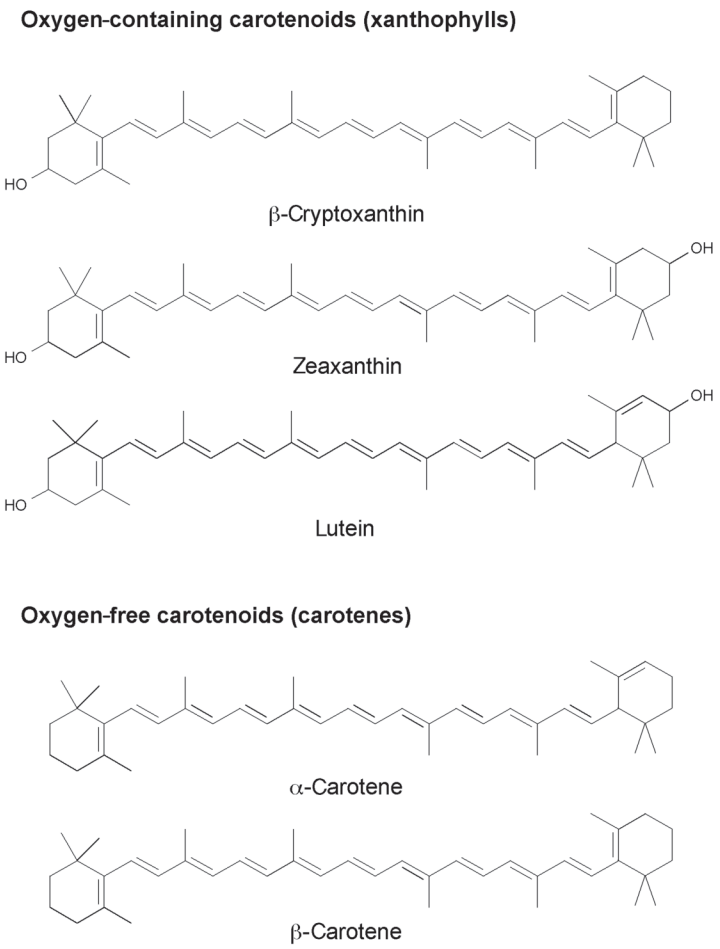

Figure 1. Structure of carotenoids: xanthophylls (oxygen-containing) and carotenes (oxygen-free).

Experimental

Materials and reagents

Acetone (analytical reagent), acetonitrile (gradient-grade), ammonium acetate (analytical reagent), chloroform (analytical reagent), ethyl acetate (analytical reagent), methanol (analytical gradient-grade), methylenechloride (analytical reagent), *n*-heptane (analytical reagent), *n*-hexane (analytical reagent), petroleum ether (40–60° fraction, analytical reagent), tetrahydrofuran (analytical reagent), and toluene (analytical reagent) were purchased from Merck (Darmstadt, Germany). Butylated hydroxytoluene (> 99% purity), lutein, β-carotene, and triethylamine (> 99% purity) were from Sigma (Deisenhofen, Germany); magnesium carbonate (analytical reagent) and the internal standard *trans*-β-apo-8'-carotenal were from Fluka (Buchs, Switzerland); and zeaxanthin and β-cryptoxanthin from Roth (Karlsruhe, Germany). Water was purified by a NanoPure unit (Barnstead, Boston, MA).

HPLC

The HPLC system used was an LC-Module I (Waters, Milford, MA), which consisted of a low-pressure gradient pump, a helium degassing system, an autosampler with a 200-µL loop, and an ultraviolet (UV)-detector (model 486, Waters). For recording UV spectra, the system was connected to a photo diode array detector (model 996, Waters) with a 10-mm pathlength flowcell. Data were recorded on a PC-based data system (Millenium, Version 32, Waters). For temperature control, a thermostatted waterbath was used.

APCI–MS coupled to LC

For HPLC–MS–MS experiments, a low-pressure gradient micropump (model Rheos 2000, Flux, Karlskoga, Sweden), a degasser (Knauer, Berlin, Germany), a microinjector (model CC00030, Valco, Houston, TX) with a 5-µL internal loop, and a variable wavelength detector (model Linear UV–vis 200, Linear Instruments, Fremont, CA) with a 1.2-µL detector cell connected to a quadrupole ion-trap MS (model LCQ, Finnigan,

Table II. Evaluation of Mobile Phase Systems for the Separation of Standard Carotenoids*

Mobile phase (v/v)	Gradient	Reference	α_1	α_2
MeOH/acetone = 95/5	isocratic	13	1.37	3.21
MeOH/THF = 95/5	isocratic	13	1.37	2.86
MeOH	isocratic	13	1.37	3.42
MeOH/ACN = 90/10	isocratic	12	1.42	3.42
MeOH/CH_2Cl_2 = 95/5	isocratic	12	1.34	2.99
MeOH/*n*-hexane = 95/5	isocratic	12	1.29	3.15
MeOH/EtOAc = 95/5	isocratic	12	1.49	3.62
MeOH/$CHCl_3$ = 95/5	isocratic	12	1.47	3.60
MeOH/Et_2O = 95/5	isocratic	12	1.33	3.12
MeOH/toluene = 95/5	isocratic	12	1.50	6.61
ACN/MeOH/CH_2Cl_2 = 75/20/5 (0.1% BHT, 0.05% TEA, MeOH 0.05M NH_4OAC)	isocratic	15	1.41	3.44
ACN/MeOH/THF = 40/55/5	isocratic	14	1.42	3.40
MeOH/ACN/$CHCl_3$/H_2O = 37/46/14/3	isocratic	24	1.55	6.07
ACN/MeOH/THF = 50/35/15	isocratic	24	1.20	6.42
MeOH/$CHCl_3$ = 90/10	isocratic	14	1.14	4.33
MeOH/THF = 90/10	isocratic	14	1.44	5.67
ACN/MeOH/CH_2Cl_2/*n*-hexane = 85/10/2.5/2.5	45/10/22.5/22.5 in 40 min	25	1.26	3.84
ACN/MeOH/CH_2Cl_2/*n*-heptane = 75/20/2.5/2.5 (0.1% BHT, 0.05% TEA, MeOH 0.05M NH_4OAC)	**isocratic**	**–**	**1.52**	**6.64**

* Macherey-Nagel Nucleosil C_{18} column (5 µm, 120Å, 250 × 3 mm).
† Lutein/zeaxanthin.
‡ Zeaxanthin/β-carotene.

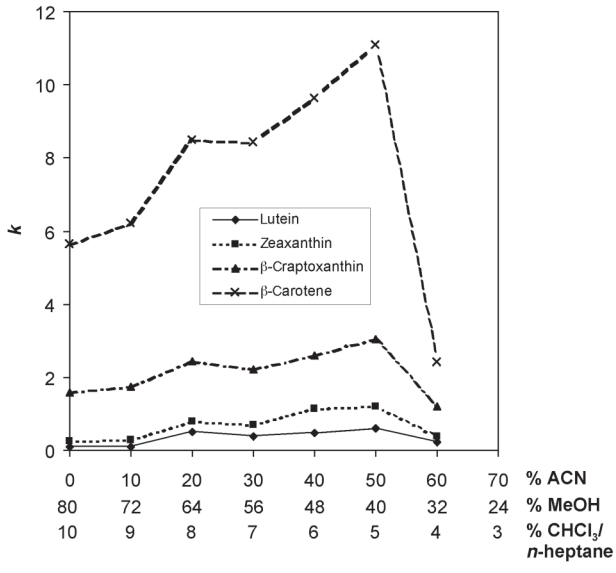

Figure 2. Dependence of capacity factor (*k*) on mobile phase composition. Column, Phenomenex Luna C_{18} (250 × 2 mm, 5 µm, 100 Å); mobile phase, ACN (0.1% BHT)/MeOH (0.05M NH_4OAc, 0.05% TEA)/$CHCl_3$ (0.1% BHT)/*n*-heptane (0.1% BHT); flow rate, 0.3 mL/min; ambient temperature; UV detection, 450 nm; sample volume, 20 µL.

San Jose, CA) were used. For APCI–MS, the following parameters were used in all experiments: temperature of the heated capillary was 150°C and source voltage was 3.97 kV. Nitrogen was used as the sheath gas (50 units).

Preparation of food items

The items were purchased at common markets in Innsbruck, Germany. Frozen materials were stored at –18°C. The other vegetables were stored in the refrigerator at 4°C for up to 4 days. The vegetable samples were prepared by the removal of the outer leaves, peeling, coring, and so forth. Immediately after preparation of the raw sample, it was frozen under nitrogen in an air-tight brown bottle at –18°C.

Sample extraction

Following the suggestions by Hart and Scott (15), the extraction procedure was carried out with modifications (24,25). Triplicate 5-g aliquots of the ground sample were placed in a conical flask together with 1 g solid magnesium carbonate to neutralize any organic acids. Fifty milliliters tetrahydrofuran and methanol (1:1, v/v) were added with the internal standard. For the extraction of the carotenoids, the vegetables were homogenized for 2 min using an Ultra-Turrax T25 homogenizer (IKA, Staufen, Germany). The resulting

suspension was filtered through a Glas Fibre filter pad (GF/A Whatman, Maidstone, U.K.) in a Buchner funnel under vacuum. The homogenizer was washed with 40 mL tetrahydrofuran and methanol (1:1, v/v). The filter pad was washed with two further aliquots of tetrahydrofuran (THF)–MeOH. The combined THF–MeOH filtrates were transferred into a separating funnel. Fifty milliliters of petroleum ether containing 0.1% butylated hydroxytoluene (BHT) and 50 mL 10% sodium chloride solution were added and mixed. The lower THF–MeOH–aqueous phase was drowned off. The upper petroleum ether phase was transferred into a 250-mL evaporating flask. The THF–MeOH–aqueous phase was extracted two more times with 50-mL aliquots of petroleum ether. The combined petroleum ether phases were combined in the flask and evaporated at 35°C to dryness. The residue was redissolved in 5 mL dichloromethane in an ultrasonic bath.

Internal standard

In order to determine the losses during the extraction procedure, *trans*-β-apo-8'-carotenal was added as an internal standard.

Preparation of standard carotenoid solutions for calibration

Accurately weighed amounts of lutein, β-carotene, and β-apo-8'-carotenal were dissolved in chloroform. The solution was filled up with *n*-hexane to give a final solvent ratio of 1:9 (v/v). β-Cryptoxanthin was dissolved in 1:1 chloroform–*n*-hexane. Zeaxanthin was dissolved in chloroform. All solvents contained 0.1% BHT. The solutions were stored in airtight screw-topped brown bottles under nitrogen at –18°C. Before preparing the working solutions, the stock solutions were brought to room temperature and filtered through a 0.2-µm membrane filter. An aliquot of the solution was evaporated under nitrogen and diluted in the appropriate solvent. The absorbance of the extract was measured on a PerkinElmer Lambda 2 UV–vis Spectrophotometer (Norwalk, CT). The concentrations were calculated using the appropriate extinction coefficients shown in Table I. Before using the working solutions, the purity was assessed by peak-purity analysis using a photo diode array detector (17).

Calculation of carotenoid content

The concentrations of carotenoids (µg/100g) were calculated using recovery factors relative to the internal standard. The recovery factor equaled the peak area of the internal standard in standard solution divided by the peak area of the internal standard after sample extraction. The content of carotenoids can then be calculated using the following equation:

$$w = [\text{RF} \times \text{amount (carotenoid in µg) in sample} \times (100\text{ g} / \text{sample size in g})] \quad \text{Eq. 1}$$

where w is the carotenoid content and RF is the recovery factor.

Results and Discussion

For the determination of the carotenoid content in selected vegetables, several mobile phase systems and HPLC columns were evaluated with respect to their selectivity and recovery. Following these preparatory works, a new mobile phase

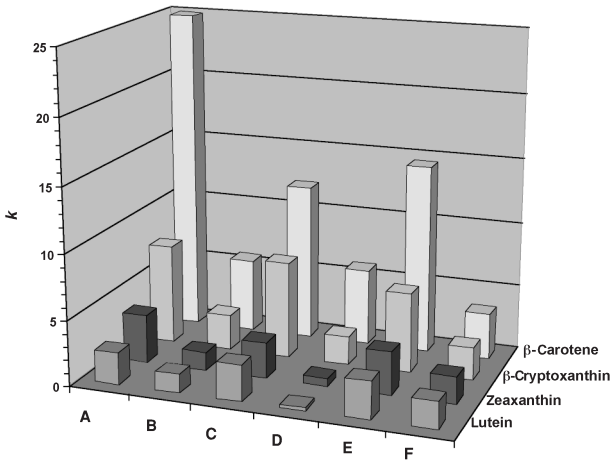

Figure 3. Capacity factors (k) of standard carotenoids using different HPLC columns. A: Phenomenex RP Si C_{18} (5 µm, 100 Å, 250 × 2 mm, flow rate 0.3 mL/min); B: Macherey-Nagel Nucleosil C_{18} (5 µm,120 Å, 250 × 3 mm, flow rate 1 mL/min); C: Techsphere ODS (5 µm, 100 Å, 250 × 4.6 mm, flow rate 1 mL/min); D: Hypersil BDS (3 µm, 130 Å, 125 × 4 mm, flow rate 1 mL/min); E: Hypersil ODS (5 µm, 120 Å, 250 × 4 mm, flow rate 1 mL/min); F: Spherisorb Octyl (5 µm, 100 Å, 250 × 4 mm, flow rate 1 mL/min). Conditions: mobile phase, ACN (0.1% BHT)/MeOH (0.05M NH_4OAc, 0.05% TEA)/$CHCl_3$ (0.1% BHT)/*n*-heptane (0.1% BHT) (50:40:5:5, v/v/v/v); ambient temperature; UV detection, 450 nm; sample volume, 20 µL.

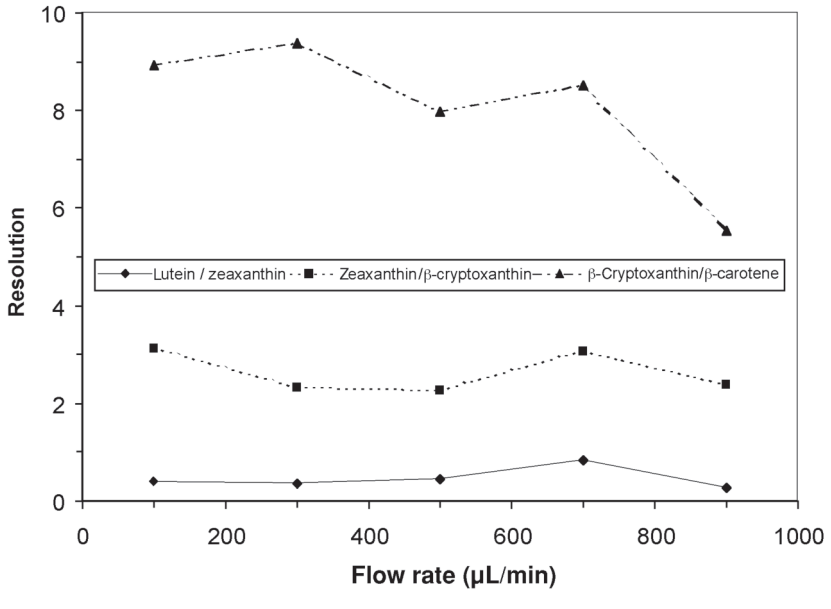

Figure 4. Dependence of resolution between standard carotenoids on flow rate. Column, Phenomenex Luna C_{18} (250 × 2 mm, 5 µm, 100 Å); mobile phase, ACN (0.1% BHT)/MeOH (0.05M NH_4OAc, 0.05% TEA)/$CHCl_3$ (0.1% BHT)/*n*-heptane (0.1% BHT) (75:20:2.5:2.5, v/v/v/v); ambient temperature; UV detection, 450 nm; sample volume, 20 µL.

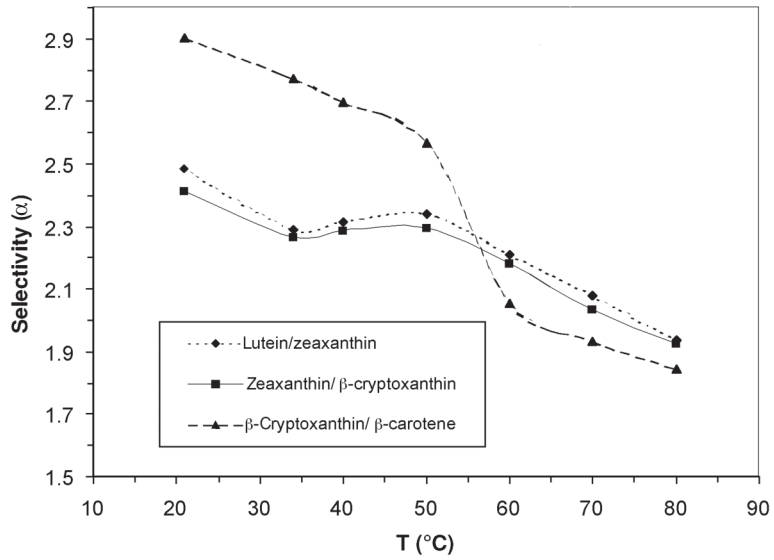

Figure 5. Dependence of selectivity (α) on column temperature. Column, Phenomenex Luna C_{18} (250 × 2 mm, 5 µm, 100 Å); mobile phase, ACN (0.1% BHT)/MeOH (0.05M NH_4OAc, 0.05% TEA)/$CHCl_3$ (0.1% BHT)/*n*-heptane (0.1% BHT) (50:40:5:5, v/v/v/v); flow rate, 0.3 mL/min; UV detection, 450 nm; sample volume, 20 µL.

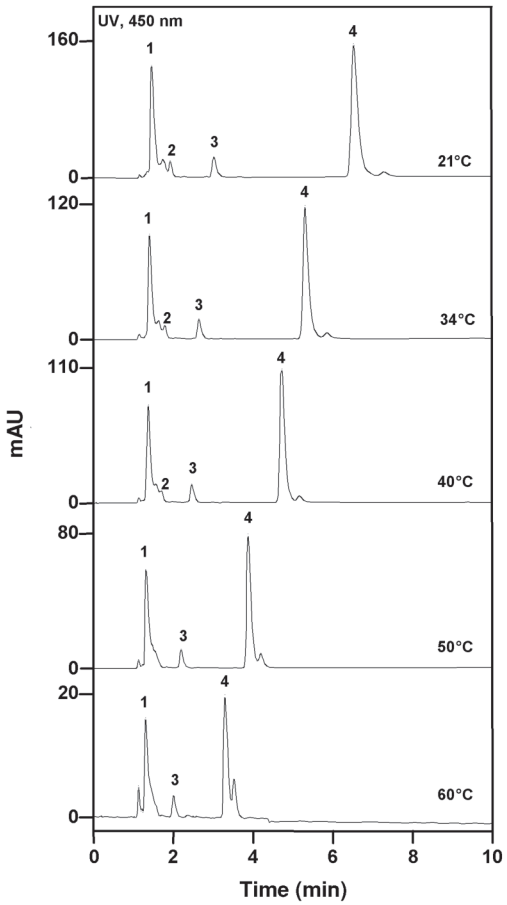

Figure 6. Separation of a standard carotenoid mixture at different temperatures: lutein, 1; zeaxanthin, 2; β-cryptoxanthin, 3; β-carotene, 4. Column and conditions are the same as that of Figure 5.

system was established that allowed for the essential improved assay for the determination of carotenoids (especially the analyte pairs lutein–zeaxanthin and β-cryptoxanthin–β-carotene) by HPLC and HPLC–MS–MS.

Evaluation of the mobile phase system

For the evaluation of mobile phase systems, the selectivity factors for lutein–zeaxanthin and zeaxanthin-β-carotene (Figure 1) were investigated. As can be deduced from Table II, the comparison study indicated that carotenoid selectivity is better using THF rather than ethyl acetate, and also better than methanol and acetonitrile. Even methanol-based solvents show higher recoveries for carotenoids than those based on acetonitrile, which correlates with the observations made by Epler et al. (8). Because carotenoids are sensitive to oxidation and may undergo losses or degradation on the stationary phase of the column, solvent modifiers should be added to the mobile phase (26). By the addition of triethylamine (TEA), unwanted silanol interactions can be suppressed (27) and the recovery can be improved (28). As TEA behaves as a strong modifier, it reduces retention time; therefore, a concentration of 0.05% should be used for the optimal resolution of carotenoids. When using chlorinated solvents, acid contamination can be associated with carotenoid losses (29). The addition of 0.05M ammonium acetate (AA) to the methanol alone provides sufficient buffer capacity (assuming that acidity is the critical factor to the recovery). When a mixture of lutein, zeaxanthin, β-cryptoxanthin, and β-carotene are chromatographed with 0.05M AA and 0.05% TEA, their recovery is improved from 70% to 95%.

Following the works published by Snyder et al. (30,31), a new quaternary mobile phase system was established in order to improve the recoveries and selectivities for the separation of carotenoids. Therefore, four organic solvents were chosen that belong to different selectivity groups: acetonitrile belongs to the selectivity group VIb (polar basis), methanol to group II (polar basis), chloroform to group VIII (nonpolar modifier), and *n*-heptane to –b (nonpolar modifier). These nonpolar modifiers basically act as a solubilizer for the applied system. Methanol acts as a stronger solvent than acetonitrile and decreases retention of all standard carotenoids, even though the latter interacts stronger with the polyene chain of carotenoids. This composition of the mobile phase ensured high selectivity within the solvent selectivity triangle (30). In order to ensure the best recoveries as described in the literature (27,29), 0.05% TEA (v/v) and 0.05M ammonium acetate were added to the methanol. In the next step, the composition of the mobile phase was optimized. As can be deduced from Figure 2, highest values for the capacity factors for the sepa-

ration of the standard mixture of lutein, zeaxanthin, β-cryptoxanthin, and β-carotene were yielded at our suggested mobile phase composition of 50% acetonitrile, 40% methanol (containing 0.05% TEA and 0.05M AA), 5% chloroform, and 5% *n*-heptane. The reason for this could be found in the optimal proportion for the solute solubility and the solvent strength.

Evaluation of HPLC columns and chromatographic conditions

The optimized mobile phase system was used to evaluate different RP-HPLC columns for the separation of the standard carotenoid mixture (lutein, zeaxanthin, β-cryptoxanthin, and β-carotene). Best capacity factors were yielded using a Phenomenex RP Si C_{18} column (5 µm, 100 Å, 250 × 2 mm) (Figure 3). When the pore size and the column's inner diameter are increased, the amount of modifier has to be decreased (32). The choice of modifier does not influence selectivity significantly. For stationary phases with better selectivity, small porosity is preferred. Following these results, the flow rate for the separation of carotenoids on the Phenomenex column was optimized. As can be deduced from Figure 4, the resolution was not significantly influenced by the flow rate. For further separation studies, a flow rate of 0.3 mL/min was used, which yielded an acceptable column back pressure.

The effect of column temperature on the separation of lutein, zeaxanthin, β-cryptoxanthin, and β-carotene was studied next. The column temperature was varied between 21°C and 80°C; the best selectivity being achieved at 21°C (Figure 5). As shown by Scott and Hart (16), it is important to work at very constant temperature, because small changes in the ambient temperature can cause significant changes in the chromatographic selectivity of the carotenoids. As can be deduced from Figure 6, when already at a temperature of 34°C, zeaxanthin could not be easily separated from lutein. At temperatures higher than 60°C, the investigated carotenoids unfortunately were not stable.

For column retention reproducibility, a test of the stability of the retention times of the standard carotenoids are shown for seven consecutive injections in Figure 7. The relative standard deviations are 1.8% for lutein, 1.8% for zeaxanthin, 2.4% for β-cryptoxanthin, and 3% for β-carotene.

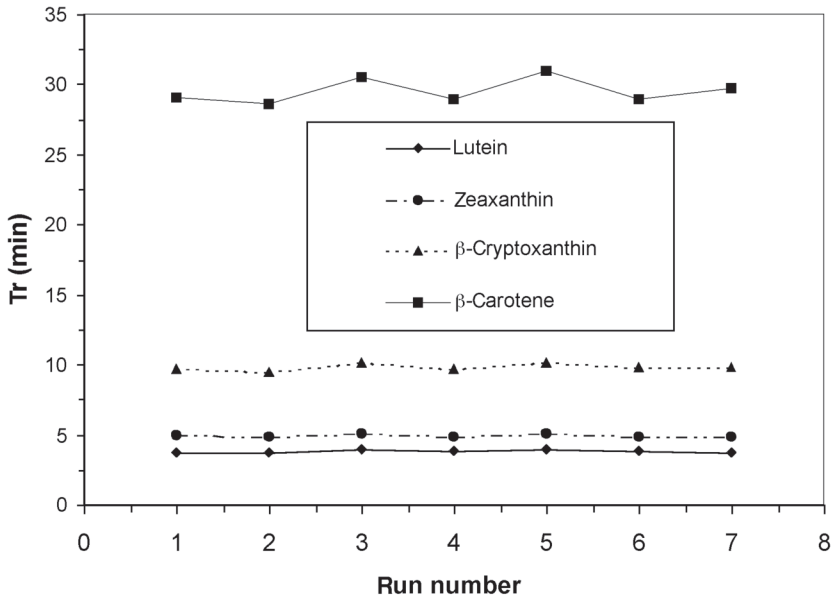

Figure 7. Reproducibility of the established chromatographic system with dependence of the retention time on the run number. Column and conditions are the same as that of Figure 5.

Table III. Calibration Curves, Regression Coefficient, and Detection Limits for the Determination of Carotenoids Using UV-Absorbance Detection at 450 nm

Carotenoid	Calibration curve	R^2	Lower detection limit*	Upper detection limit*
Lutein	$y = (6 \cdot 10^6)x - 181370$	0.992	26.1 pg	13.1 µg
Zeaxanthin	$y = (3 \cdot 10^7)x - 3318.6$	0.989	1.04 pg	7.8 g
β-Cryptoxanthin	$y = (3.05 \cdot 10^7)x + 10^6$	0.965	0.208 pg	8.3 µg
β-Carotene	$y = 10^7x - 341876$	0.987	0.938 pg	6.3 µg

*(µV • seconds – µg)

Quantitation of carotenoids in selected vegetables

Probable sources of error and variance both within and between laboratories usually is in the preparation and calibration of stock and working standard carotenoid solutions and their use in quantitating the carotenoid content of samples. Therefore, the solubility (dissolution) of carotenoids must be ensured, even if all solutions are filtered before the establishing of concentrations by absorbance at a specified wavelength. Each time before a working solution is prepared, the accuracy of the concentration of the standard solution should be ensured by measuring the absorption. The plots of peak area versus concentration for lutein, zeaxanthin, β-cryptoxanthin, and β-carotene recorded by UV detection at 450 nm showed good linearity, whereas β-cryptoxanthin showed the smallest correlation coefficient (R^2 = 0.965) (Table III). The lower detection limit was found in the picogram range and the upper detection limit was found in the microgram range for all investigated carotenoids. These calibration curves were used for the quantitation of lutein, zeaxanthin, β-cryptoxanthin, and β-carotene in carrot, spinach, and kale (Figure 8) and celery, broccoli, and various tomato preparations (flakes, granulate, and powder) (Figure 9). Good sources for lutein are spinach, kale, and broccoli, and sources for β-carotene are kale, broccoli, spinach, carrots, and tomatoes (Table IV). The found

values correlated well with those of the Nutrition Table published by Souci, Fachmann, and Kraut (33). It should be noted that the content of particular items may be affected by variety, maturity, growing conditions, the season of the year, and which part of the item is consumed. In general, outer parts of plants contain higher levels than inner parts.

LC–MS of carotenoids

After the quantitation of carotenoids in selected vegetables, HPLC–MS–MS was used to ensure correct peak identification and purity. Most publications dealing with the coupling of HPLC to the MS have used an ESI (18–21). In comparison with the ESI (which ionizes the sample in a powerful electrical field), APCI facilitates ionization by classical chemical reactions such as proton transfer. Under these conditions, xanthophylls and carotenes form ions during either positive- or negative-ionization in LC–MS. The sensitivity of this technique has been shown to be similar to that of electrospray-MS (18).

The mechanism of the formation of unusual protonated hydrocarbons such as xanthophylles and carotenes is under investigation. One possible explanation for the formation of protonated carotene molecules may be that π-electrons of the carotenoid polyene chain are promoted to various excited states through interaction with the APCI plasma so that one or more of these excited-state carotenoids exhibits unusually high proton affinity compared with the ground-state molecule. This increased proton affinity may result in the protonation of the carotene by the APCI solvent as reagent gas.

Table V shows that APCI produces a linear detector response/concentration curve with correlation coefficients greater than 0.988 over sample concentrations of at least 3 orders of magnitude. Therefore, APCI is an excellent technique for the quantitation of carotenoids in complex sample matrices during LC–MS. Because of the fact that the mobile phase used contained 0.1% butylated hydroxytoluene, an AA–TEA buffer system, and chloroform as a part of the mobile phase (which had direct influence in the ionization proce-

Table IV. Carotenoid Content of Vegetables*

Vegetable	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene
Broccoli	2358	–	30	925
Carrot	280	–	traces	4881
Celery	–	–	–	33
Kale	6390	62	–	1500
Savoy	–	–	–	54
Spinach	3920	130	–	4650
Tomato flakes	99	–	–	397
Tomato granulate	226	–	–	829
Tomato powder	39	–	–	726

* (µg/100 g "wet weight" as eaten), mean value of 3 samples.

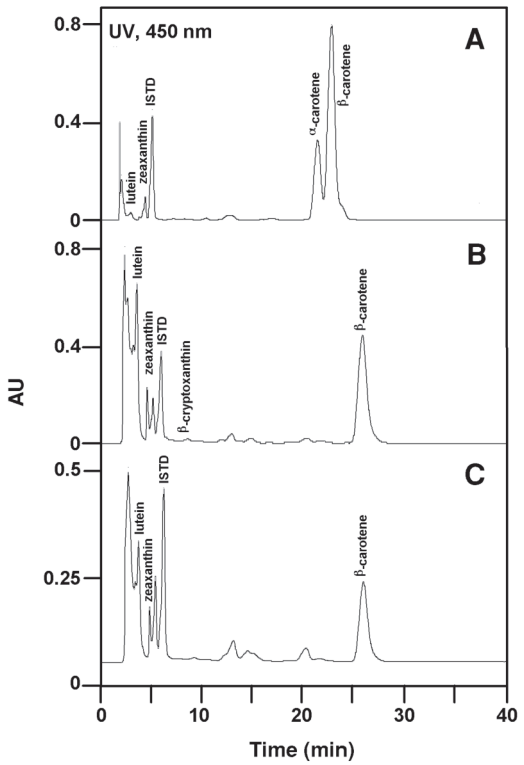

Figure 8. HPLC determination of carotenoids in carrots, A; spinach, B; and kale, C. Column and conditions are the same as that of Figure 5.

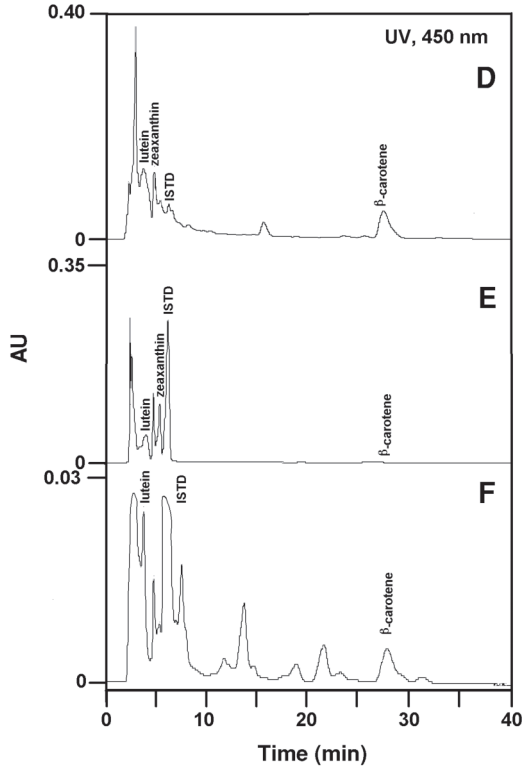

Figure 9. HPLC determination of carotenoids in tomato powder, D; celery, E; and broccoli, F. Column and conditions are the same as that of Figure 5.

Table V. Calibration Curves, Regression Coefficient, and Detection Limits for the HPLC Determination of Carotenoids Using APCI–MS Detection*

Carotenoid	Calibration curve	R^2	Lower detection limit
Lutein	$y = 217049x - 13115$	0.991	12.5 ng
Zeaxanthin	$y = 460837x + 931.98$	0.996	1.2 ng
β-Cryptoxanthin	$y = 148291x - 23.173$	0.997	8.7 ng
β-Carotene	$y = 493623x - 18.374$	0.988	15.1 ng

* (area – ng).

Table VI. Molecular Weight and Characteristic Fragments of Carotenoids

Carotenoid	Molecular weight	Most abundant fragments
Lutein, zeaxanthin	569	551, 459, 429
β-Cryptoxanthin	553	535, 459
β-Carotene	537	480, 444, 388

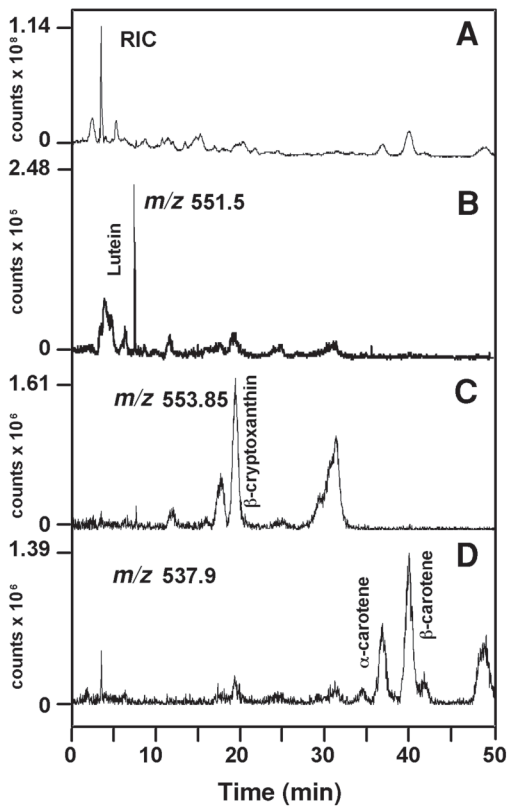

Figure 10. HPLC–APCI–MS of a carrot extract. Column, Phenomenex Luna C_{18} (250 × 2 mm, 5 µm, 100 Å); mobile phase, ACN (0.1% BHT)/MeOH (0.05M NH_4OAc, 0.05% TEA)/$CHCl_3$ (0.1% BHT)/*n*-heptane (0.1% BHT) (50:40:5:5, v/v/v/v); flow rate, 0.2 mL/min; ambient temperature. A: APCI–MS, total ion chromatogram, scan 300–2000 amu in 1 s; B: APCI–MS, trace of *m/z* 551.5; C: APCI–MS, trace of *m/z* 553.85; D: APCI–MS, trace of *m/z* 537.9; sample volume, 5 µL.

dure), the lower detection limit for lutein, zeaxanthin, β-cryptoxanthin, and β-carotene was found in the lower nanogram range.

Figure 10 shows the analysis of a carrot extract using positive-ion APCI. A number of unresolved peaks are seen in the total ion chromatogram (Figure 10A). In contrast to the other carotenoids, the hydroxylated xanthophyll lutein showed a base peak at *m/z* 551, corresponding to the loss of water from the protonated molecule. The extraction of a selected ion chromatogram at *m/z* 551.5—the $[MH–H_2O]^{+\bullet}$ peak in the spectrum of lutein—showed one large peak at 8.1 min and several small peaks in the rest of the chromatogram (Figure 10B). The extraction of selected ion chromatograms at *m/z* 553.85 and *m/z* 537.9 allowed for the determination of β-cryptoxanthin (Figure 10C) and α- and β-carotene (Figure 10D). Generally, the determination of carotenoids in vegetable samples allowed for a more efficient determination, because the peaks of interest were base-line separated in the extracted ion chromatograms. In order to enhance carotenoid fragmentation, CID was used to produce significant carotenoid fragment ions. This technique can also be used as a fingerprint for determination in very complex sample matrices, whereas the fragmentation patterns of carotenoids depend on the choice of the used interface (Table VI). The positive-ion CID mass spectrum of lutein showed fragment ions of *m/z* 551, 459, and 429, which correspond to the loss of water, loss of water and toluene, and loss of the terminal ring. Zeaxanthin gives the same fragmentation pattern with slightly different abundances as lutein and has to be distinguished from lutein by its retention time. The most abundant fragments for β-cryptoxanthin were the loss of water (*m/z* 535) and the loss of toluene (*m/z* 459). The dissociation of β-carotene showed fragments at *m/z* 480 (retro-Diels-Alder fragment ion), 444 (loss of toluene), and 388 (combination of loss of toluene and retro-Diels-Alder fragmentation).

Conclusion

The presented results indicate that the optimization of a new mobile phase system—ACN (0.1% BHT)/MeOH (0.05M NH_4OAc, 0.05% TEA)/$CHCl_3$ (0.1% BHT)/*n*-heptane (0.1% BHT)—on a Phenomenex Luna Si C_{18} column (250 × 2 mm, 5 µm, 100 Å) allows a more efficient separation of carotenoids compared with previous reports. UV-absorbance detection allows the determination of the carotenoids down to the picogram range with good linearity. For the determination of carotenoids in complex sample matrices, the coupling of the HPLC to an MS using an atmospheric pressure interface is a useful tool. Furthermore, CID in the ion source allows for the identification of carotenoids because of their characteristic fragmentation pattern. The introduced method can be used to detect carotenoids in complex biological tissues with improved recovery and selectivity.

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