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Separation and identification of various carotenoids by C_{30} reversed-phase high-performance liquid chromatography coupled to UV and atmospheric pressure chemical ionization mass spectrometric detection

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Abstract

In this paper the application of on-line HPLC–UV–APCI (atmospheric pressure chemical ionization) mass spectrometry (MS) coupling for the separation and determination of different carotenoids as well as *cis*/*trans* isomers of β-carotene is reported. All HPLC separations were carried out under RP conditions on self-synthesized polymeric C_{30} phases. The analysis of a carotenoid mixture containing astaxanthin, canthaxanthin, zeaxanthin, echinenone and β-carotene by HPLC–APCI-MS was achieved by scanning the mass range from *m*/*z* 200 to 700. For the characterization of a sample containing *cis*/*trans* isomers of β-carotene as well as their oxidation products, a photodiode-array UV–visible absorbance detector was used in addition between the column and the mass spectrometer for structural elucidation of the geometrical isomers. The detection limit for β-carotene in positive-ion APCI–MS was determined to be 1 pmol. In addition, an extract of non-polar substances in vegetable juice has been analyzed by HPLC–APCI-MS. The included carotenoids could be identified by their masses and their retention times.

Keywords: Vegetable juices; Food analysis; Carotenoids; Terpenes

1. Introduction

Carotenoids are naturally-occurring tetraterpenes found in various fruits, vegetables, plants, algae and bacteria. An important structural feature of these compounds is the existence of a highly conjugated system of double bonds resulting in *cis* and *trans* isomers [1]. Carotenoids are not only essential for human health but also show promising effects as preventive and therapeutic agents. Studies have proven that consumption of β-carotene, lycopene, and other carotenoids decreases the risk of degenerative diseases, such as certain kinds of cancer and cardiovascular disease. Additionally, they have been suggested to enhance the immune response. Carotenoids also play an important role as antioxidants due to their ability to quench the highly damaging singlet oxygen, and to scavenge free radicals in vivo [2]. Carotenoids exhibit bioactivity as precursors of provitamin A and play an important role in the vision process and for the prevention and treatment of age-related macular degeneration [3].

The separation of either different carotenoids or *cis*/*trans* isomers of a special carotenoid is therefore

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of common interest. For this purpose, a polymeric C_{30} phase was developed by Sander [4], which is more effective for the separation of non-polar geometric isomers than the commercially available C_{18} phases [5–8].

For a fast and unambiguous structural assignment, the coupling hyphenation of chromatography to mass spectrometry is an elegant solution. But since carotenoids belong to a rather non-polar substance class, their ionization for mass spectrometric detection is not simple. Since they lack a site for protonation, detection by ESI (electrospray ionization) is difficult. Therefore, carotenoids have been investigated by the use of particle beam [9,10] or APCI (atmospheric pressure chemical ionization) MS [11,12]. For ESI, good results have been obtained by the use of additional chemicals which facilitate the ionization process, such as, e.g., halogen-containing eluents [13,14], silver salts [15] or ferrocene-based derivatives [16].

2. Experimental

2.1. Chemicals

Samples of pure all-*trans* compounds of β-carotene, lycopene, astaxanthin, canthaxanthin, zeaxanthin, echinenone were a gift from Hoffmann-La Roche. Methanol and methyl *tert*.-butyl ether (MTBE) were of Gradient-Grade quality (Merck, Darmstadt, Germany). The investigated vegetable juice was made of a mixture of tomatoes, carrots, spinach and celery and was a common brand available in any supermarket.

2.2. Extraction and sample preparation

For extraction of carotenoids from the vegetable juice, 2 ml of juice were thoroughly mixed with 1 ml of MTBE. After phase separation of the two liquids, the organic phase was directly injected for analysis

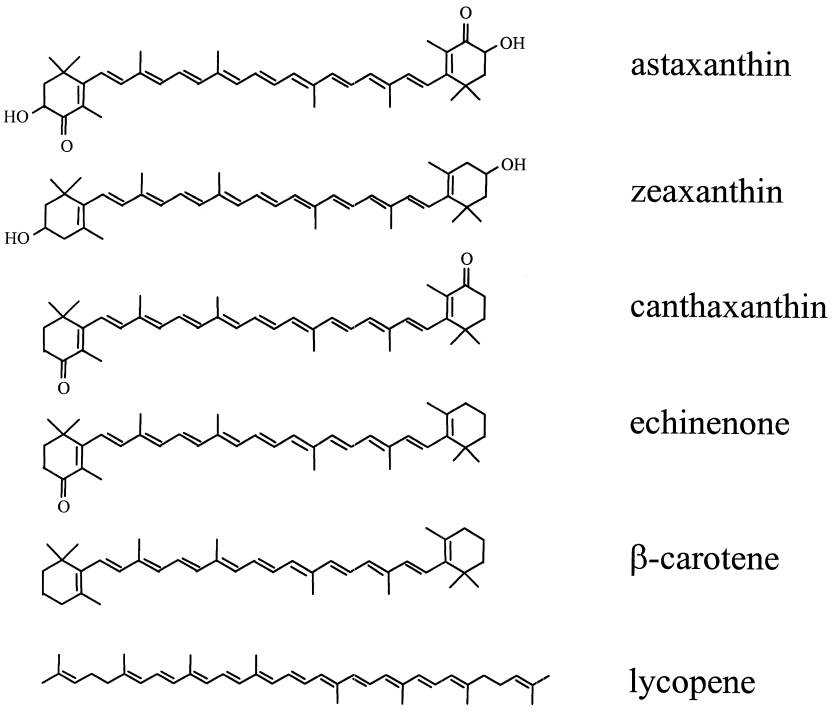

Fig. 1. Structures of different all-*trans* carotenoids including the xanthophylls astaxanthin, zeaxanthin, canthaxanthin and echinenone and the carotenes β-carotene and lycopene.

without further sample preparation. Carotenoids have been dissolved in MTBE and stored in the dark. For the isomerization of all-*trans* β-carotene, 2 ml of solution was mixed with one drop of iodine/hexane (about 5 mg/ml) and exposed to sunlight for 6 h.

2.3. Chromatography

For chromatographic separations an HP 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode-array detector and an automatic sample injector has been used. All separations have been performed with a 250 mm×4.6 mm column packed with ProntoSil silica gel (3 μm, 200 Å, Bischoff, Leonberg, Germany) modified with triacontyltrichlorosilane (C_{30}, ABCR, Karlsruhe, Germany) as stationary phase. The modification procedure followed the protocol previously published [4,5]. Chromatography was carried out in methanol–MTBE with a flow-rate of 1 ml/min and was monitored at 450 nm. The injection volume was 10 μl of the prepared MTBE solutions. All UV spectra were recorded in the range from 320 to 700 nm and acquired by ChemStation software system (Hewlett-Packard).

2.4. Mass spectrometry

Mass spectra were monitored in the mass range *m/z* 200–700 (detection limit, carotenoid mixture, vegetable juice) and *m/z* 80–1000 (β-carotene isomers) on a LCQ mass spectrometer equipped with an APCI interface (Finnigan MAT, Bremen, Germany). The capillary temperature was set to 150°C, the APCI vaporizer temperature was held at 450°C. The corona discharge voltage was optimized to 8 kV resulting in a current of 5 μA. Sheath gas flow and auxiliary gas flow (both nitrogen) were set to 70 and 30, respectively (arbitrary units). The spectrometer has been tuned to optimize the signal of *m/z* 537 ($[M+H]^{\oplus}$ of β-carotene). Detection was performed in the positive mode with an ion injection time of 5 ms and the accumulation of 3 microscans for each scan with a maximum ion time of 400 ms for each mass spectrum.

3. Results and discussion

For the separation and identification of various carotenoids shown in Fig. 1, *cis/trans* isomers of β-carotene and their oxidation products, HPLC–UV–APCI-MS coupling has been performed.

The limits of detection for β-carotene using positive-ion APCI-MS was determined by flow injection using full-scan detection in the range of *m/z* 200–700 and by extraction of the mass *m/z* 537 of the protonated molecule $[M+H]^{\oplus}$. After serial dilutions of all-*trans* β-carotene, 10 μl aliquots of each standard were injected onto the column, eluted with methanol–MTBE (70:30, v/v) and transferred

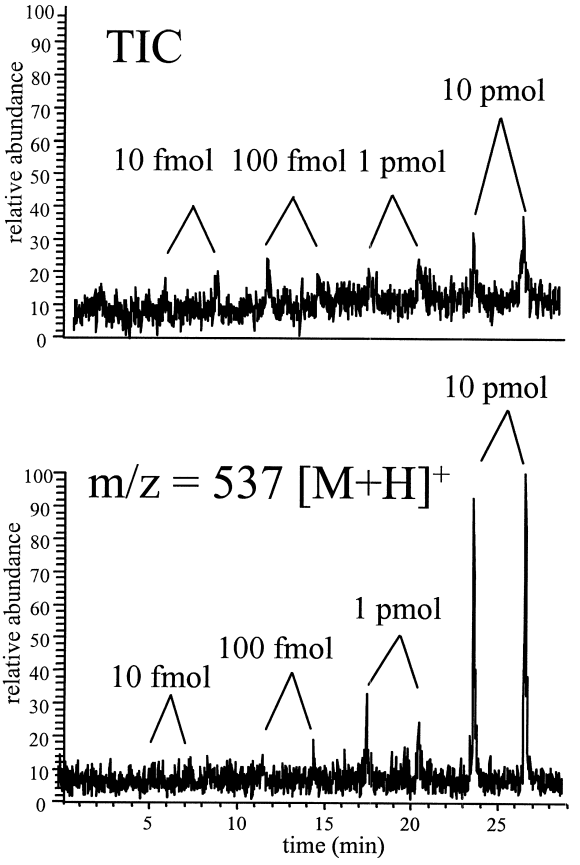

Fig. 2. Detection limit of β-carotene in positive-ion APCI-MS using full-scan detection in the range of *m/z* 200–700 (top) and by extraction of the mass *m/z* 537 of the protonated molecule $[M+H]^{\oplus}$ (bottom).

to the mass spectrometer at a flow-rate of 1 ml/min. We determined a detection limit of 1 pmol (0.54 ng absolute amount or 45 pg/μl) (Fig. 2). To classify the detection limit obtained in our laboratory, a comparison with published data is given: Van Breemen et al. determined the detection limit for α-carotene using either positive- or negative-ion APCI-MS to 3.5 pmol [11] and for β-carotene using ESI with the addition of halogenated solvents to 1–2 pmol [13]. Using APCI-MS detection, a detection limit for β-carotene of about 1 ng/μl has been published by Clarke et al. [12]. By adding silver-ions to the chromatographic eluent prior to ESI-detection, a detection limit of 3 pmol could be achieved [15]. Since the determined detection limit is below the previously published, the combination of C_{30}-HPLC with APCI-MS detection is a fast and elegant method for the detection of carotenoids down to a few ng/ml concentration without modification of the separation process or the use of halogenated solvents.

The recorded mass spectrum of all-*trans* β-carotene is shown in Fig. 3. The base peak at *m*/*z* 537 belongs to the protonated molecule, $[M+H]^{\oplus}$. As observed with other easily ionized compounds [16], also the molecular radical ion $[M]^{\cdot\oplus}$ with the mass *m*/*z* 536 appears with high intensity. Another ion recorded at *m*/*z* 444, identified as $[M-92]^{\cdot\oplus}$, is a typical fragment for β-carotene and it is formed by free-radical fragmentation from the radical cation $[M]^{\cdot\oplus}$.

The application of HPLC–UV–APCI-MS coupling for the identification of *cis*/*trans* isomers of β-carotene is shown in Fig. 4. The upper chromatogram depicts the UV trace recorded at 450 nm. The lower chromatogram corresponds to the total-ion chromatogram (TIC) recorded at a mass range between *m*/*z* 200 and 700. The third part of the figure shows the computer reconstructed mass chromatogram of the protonated molecule (*m*/*z* 537). By comparison of this mass extract with the UV trace, it is obvious that all compounds eluting after 10 min in the chromatogram belong to *cis*/*trans* isomers of β-carotene. The earlier eluting components belong to oxidized degradation products with a molecular mass

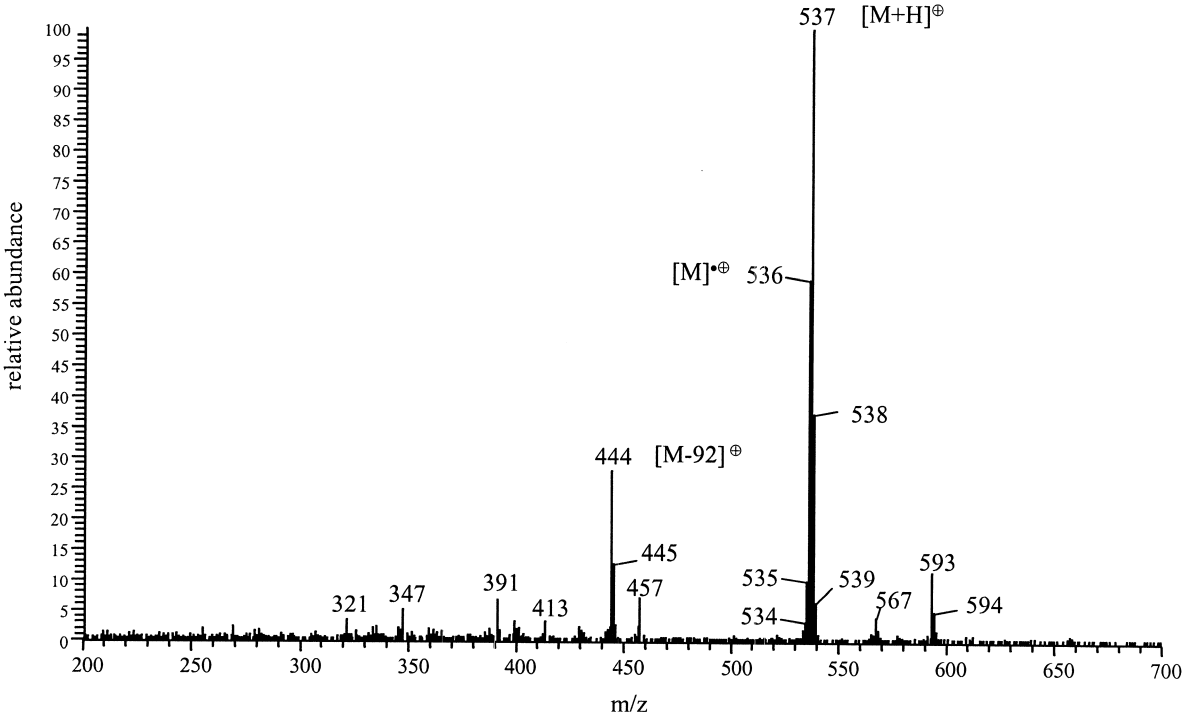

Fig. 3. Positive ion APCI mass spectrum of β-carotene.

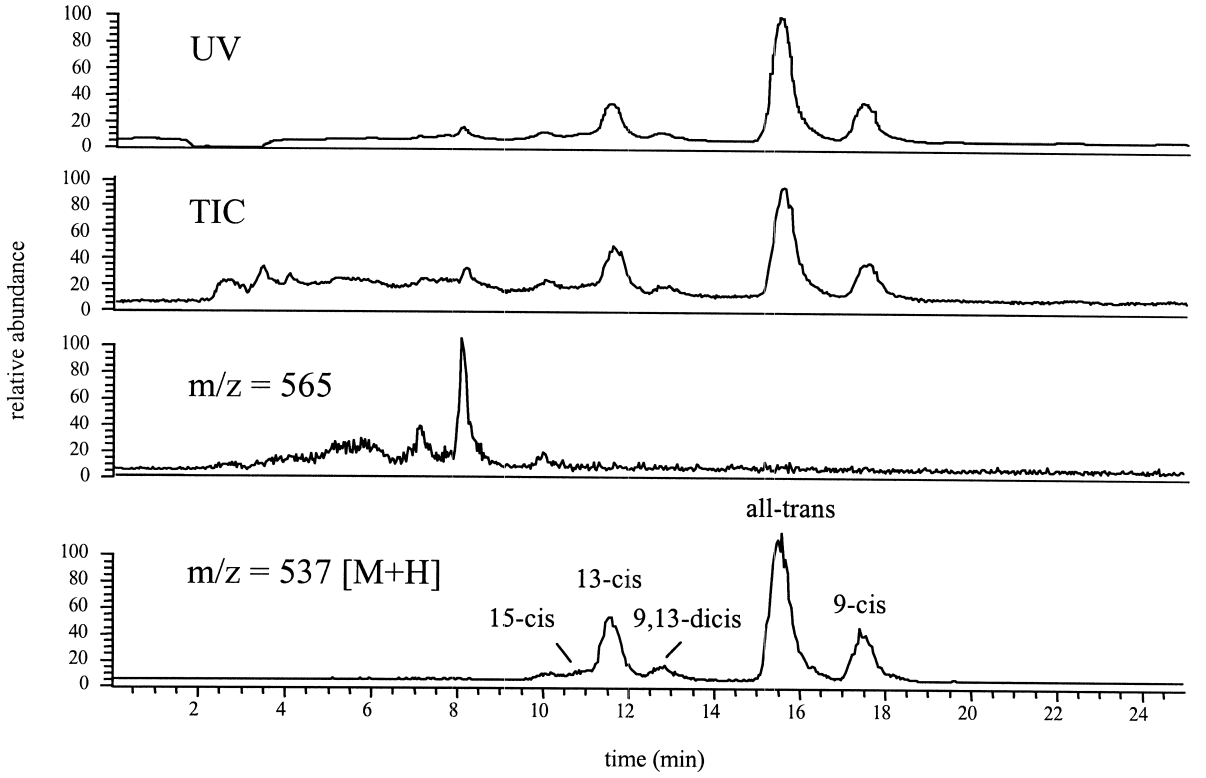

Fig. 4. HPLC–APCI-MS coupling of β-carotene using a C_{30} reversed-phase HPLC column.

of 564, as seen in the lower mass chromatogram of *m/z* 565. This corresponds to two additional carbonyl groups in the β-carotene molecule, probably at both ionone rings. Surprisingly, no peaks with oxidized β-carotene containing only one additional oxygen atom could be found.

The structural elucidation of these *cis/trans* isomers with mass spectrometry is not possible because of the identical fragmentation patterns. So, to aid structure determination, the UV spectra of the corresponding β-carotene *cis/trans* isomers were recorded at the chromatographic peak maximum during the HPLC–APCI-MS run shown in Fig. 5. The appearance of a *cis* bond in most carotenoids leads to a hypsochromic shift of λ_{max} compared to λ_{max} of the all-*trans* molecule. It is obvious, that as the *cis* bond of the isomer moves to the center of the molecule the absorption maximum of the UV spectrum shifts to shorter wavelengths and an additional band, the so called *cis* band, appears at 340 nm with increasing intensity. The 15-*cis* isomer is well characterized by the very high intensity of its *cis* band. All other isomers have been identified independently by coupled chromatography and NMR spectroscopy. An interesting feature of the depicted UV spectra is the intensity of the *cis* band of 9,13-di-*cis* molecule, which has an intensity higher than that of the 9-*cis* isomer, but remarkably lower than that of 13-*cis* β-carotene.

The application of HPLC–APCI-MS coupling for the separation and identification of a carotenoid mixture containing echinenone, canthaxanthin, astaxanthin, zeaxanthin and β-carotene has also been investigated. All substances were separated within 23 min by gradient elution on a C_{30} column. The results are shown in Fig. 6. The upper chromatogram corresponds to the UV trace which was recorded at 450 nm. The second chromatogram depicts the MS trace recorded in the full-scan mode in the mass range between *m/z* 200 and 700. The differences in retention times between the UV trace and the TIC are due to the transfer time of the eluent from the

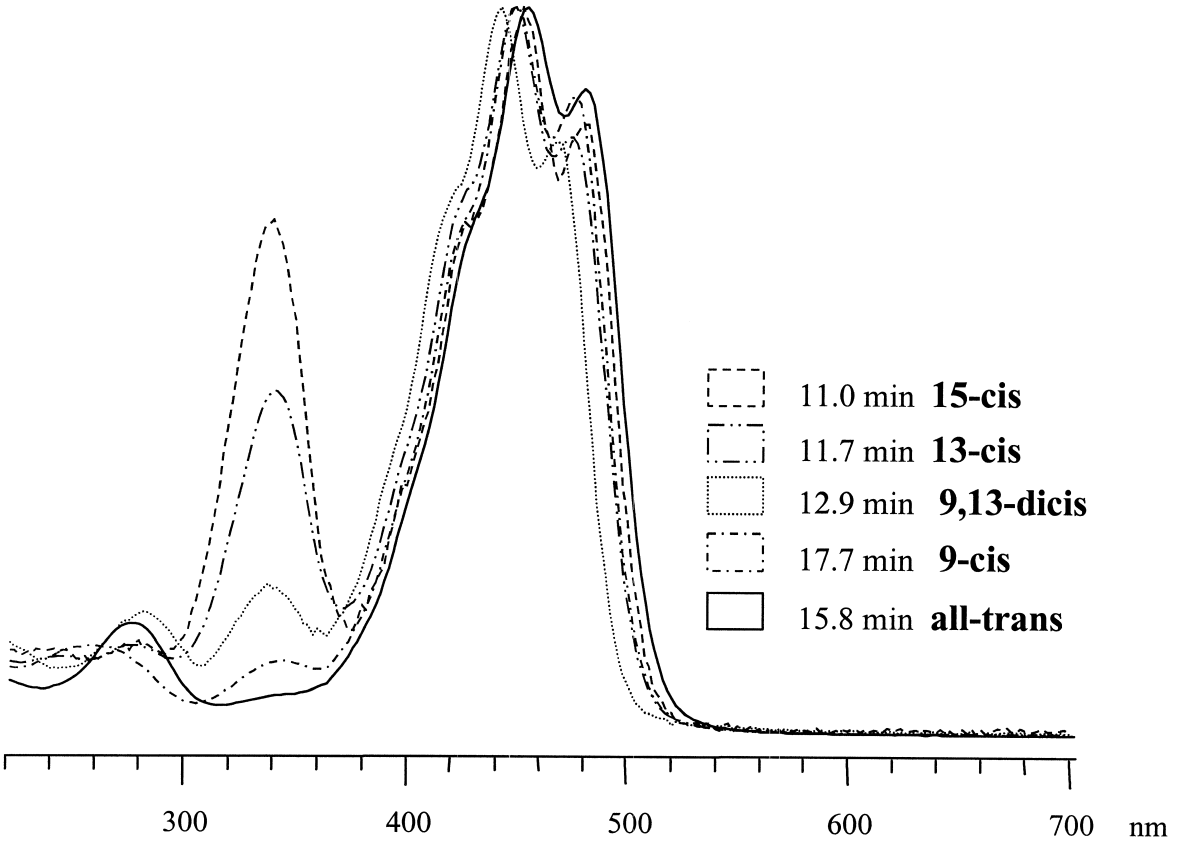

Fig. 5. UV spectra of different *cis*/*trans* isomers of β-carotene recorded on-line during the HPLC–APCI-MS analysis shown in Fig. 7 using a photodiode-array detector.

UV detector cell to the mass spectrometer. All peaks could be identified by their retention time and by their $[M+H]^{\oplus}$ signal. The other traces in Fig. 6 belong to the computer reconstructed mass chromatograms of the corresponding protonated molecules at m/z 597 for astaxanthin, m/z 569 for zeaxanthin, m/z 565 for canthaxanthin, m/z 551 for echinenone and m/z 537 for β-carotene. By comparing the extracted mass chromatograms with the UV trace and the TIC, all substances could be identified.

We wanted to test the above method with a real-life sample, where unexpected matrix effects could affect the ionization process. For the identification of carotenoids in vegetable juice, we extracted all non-polar substance with MTBE and used this solution without further sample treatment. The separation of the different compounds in the extract could be achieved by gradient elution within 70 min as shown in Fig. 7. The sample contained a high amount of one substance dominating the UV chromatogram and also some other non-polar substances which gave smaller peaks. Since the UV chromatogram was recorded at 450 nm, there is a high possibility that these peaks belong to some carotenoids. By extracting the mass m/z 537, which represents the protonated molecule with the molecular mass for β-carotene as well as for lycopene, and comparing it with the UV chromatogram, it is obvious that this juice contains a large amount of lycopene and also a small amount of β-carotene. Fig. 7 also depicts the mass spectrum of lycopene, which has been extracted from the maximum of the peak eluting at 67

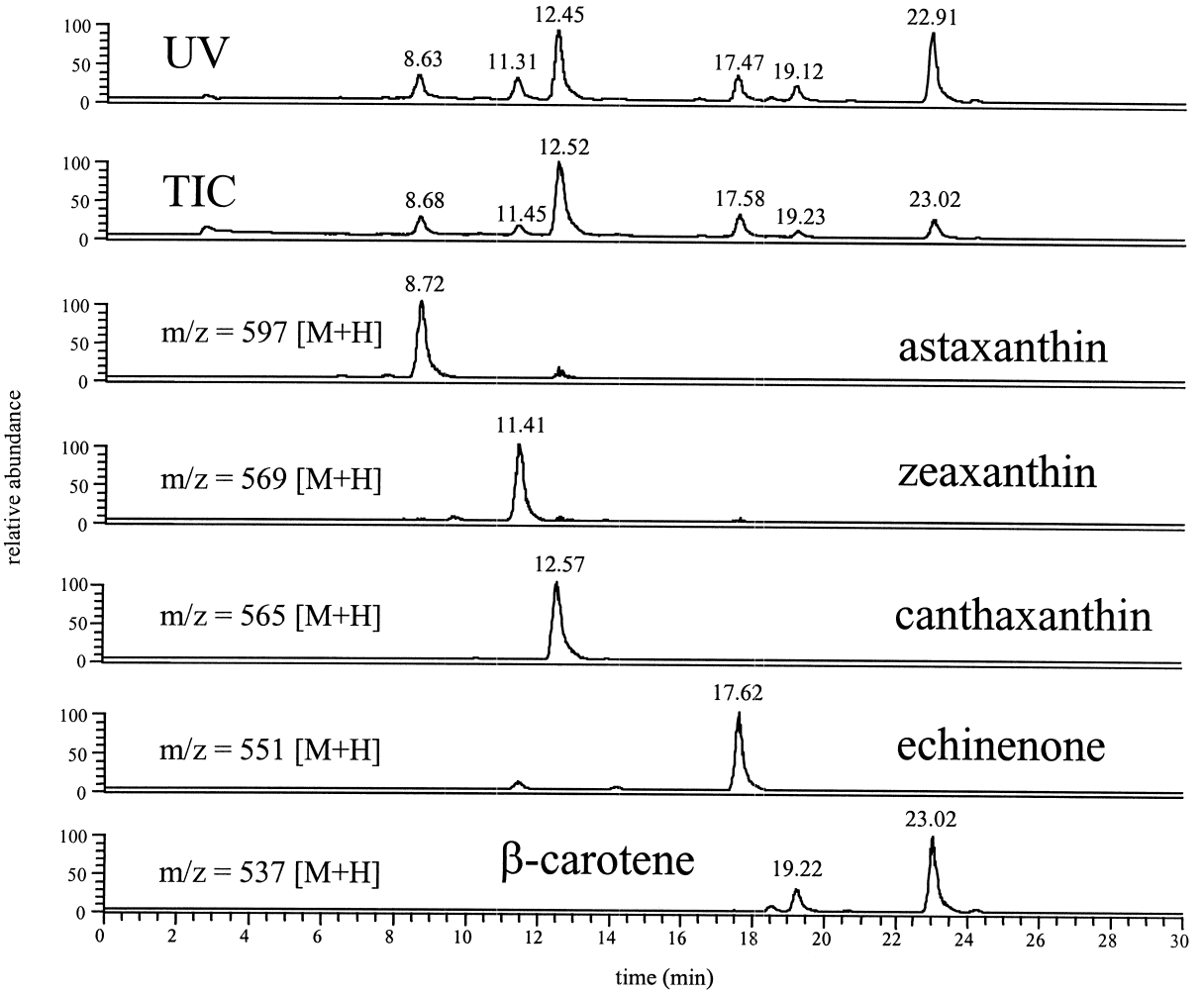

Fig. 6. HPLC–APCI-MS analysis of a mixture of different carotenoids using a C_{30} reversed-phase HPLC column.

min. Similarly to the mass spectrum of β-carotene, the base peak at m/z 537 belongs to the protonated molecule, the $[M+H]^{\oplus}$-ion, but also the molecular radical ion $[M]^{\cdot\oplus}$ with m/z 536 and the $[M-92]^{\cdot\oplus}$ radical cation at m/z 444 appear with high intensity.

4. Conclusions

In our study we proved that the developed method is not only suitable for the investigation of carotenoids in purified solutions but is also useful for carotenoids in complex samples (e.g. in food samples, without extensive sample pretreatment prior to the HPLC–APCI-MS analysis. The use of C_{30}-RP phases for the separation of carotenoids leads to a good separation and short analysis times and results in sharp peaks which facilitate good detector response. Using APCI-MS detection, additional modification of the chromatographic eluent is not necessary, which yields a robust method that is easily implemented into standard analytical systems.

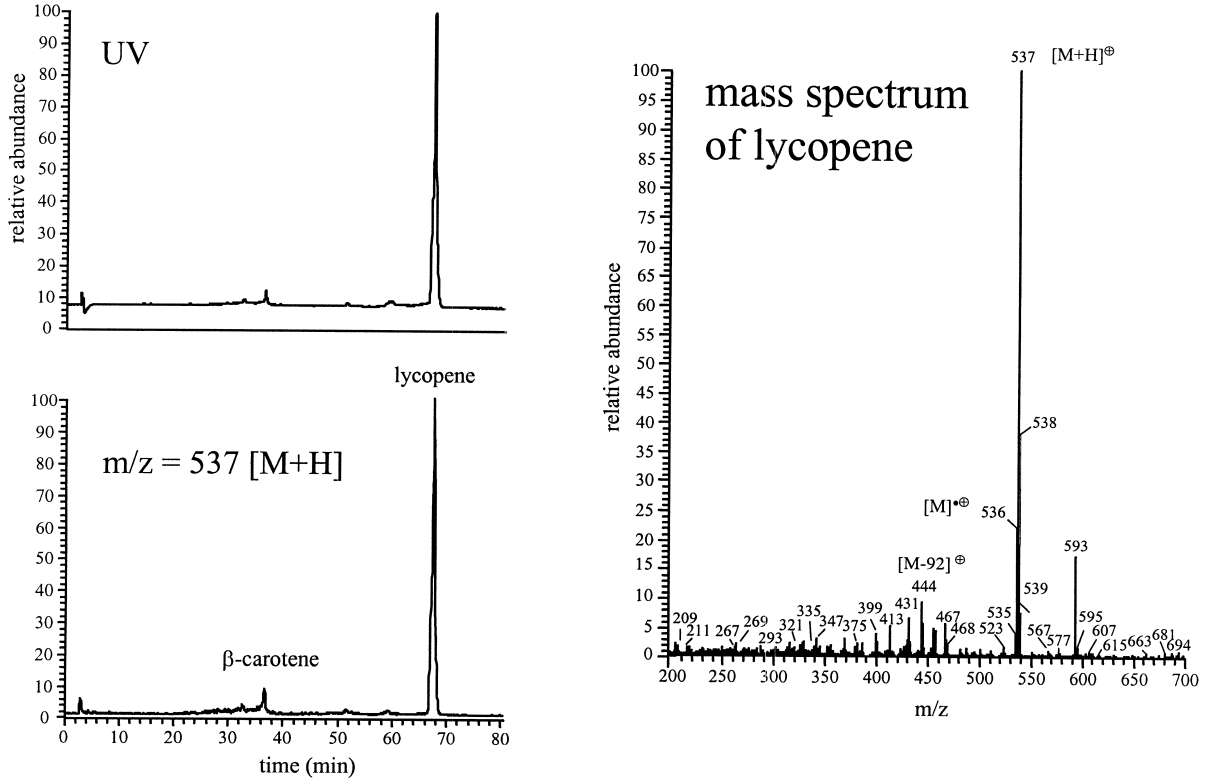

Fig. 7. HPLC–APCI-MS analysis of a vegetable juice using a C_{30} reversed-phase HPLC column; left: absorbance chromatogram at 450 nm and computer reconstructed mass chromatogram of m/z 537 corresponding to the mass of β-carotene and lycopene; right: mass spectrum of lycopene extracted at the maximum of the peak eluting at 67 min.

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