ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 669–677

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Hyphenation of high performance liquid chromatography with nuclear magnetic resonance spectroscopy for the characterization of β-carotene isomers employing a C_{30} stationary phase☆

Sabine Strohschein, Matthias Pursch, Klaus Albert *

Universität Tübingen, Institut für Organische Chemie, Auf der Morgenstelle 18, D-72076 Tübingen, Germany

Received 15 May 1998; received in revised form 26 February 1999; accepted 15 March 1999

Abstract

The hyphenation of HPLC together with NMR spectroscopy proves advantageous for the structure elucidation of oxidation- and UV-sensitive compounds such as β-carotene isomers. In the closed-loop HPLC–NMR system, degradation or isomerization of separated compounds is largely hindered. With the help of 3-μm C_{30} stationary phases a better separation efficiency towards the different β-carotene *cis/trans* isomers could be obtained in comparison to a 5-μm material, resulting in sharper peaks and a better resolution of all compounds. This effect greatly faciliated the structure determination of the isomers by HPLC–NMR coupling. Due to the introduction of a superior stationary phase, the structure of seven *cis*-isomers of β-carotene could thereby be determined employing the stopped-flow HPLC–^{1}H-NMR mode.

Keywords: HPLC–NMR coupling; Stopped-flow HPLC–^{1}H NMR mode; 2-D NMR spectroscopy; Separation of β-carotene isomers; C_{30} stationary phase

1. Introduction

The structural assignment of unknown compounds separated by a chromatographic separation technique is often a problem. Nuclear magnetic resonance (NMR) spectroscopy with its superior stereochemical information content can be used as a universal detection technique for structural elucidation. Because of the relatively low sensitivity of ^{1}H-NMR spectroscopy for separation with analytical columns, 120-μl NMR flow-cells are mainly used. For the registration of either 1-D or 2-D ^{1}H-NMR spectra in an on-line HPLC–NMR coupling experiment, it is crucial to reach the highest possible concentration level of every separated compound in the NMR flow-cell. At least the apparent concentration of the peak of interest should fit to the currently available NMR sensitivity levels in the upper nanogram range.

☆ Presented at the Drug Analysis '98 meeting, Brussels, Belgium, 11–15 May 1998.

* Corresponding author. Tel.: +49-7071-297-5335; fax: +49-7071-295-875.

E-mail address: klaus.albert@uni-tuebingen.de (K. Albert)

0731-7085/99/$ - see front matter
PII: S0731-7085(99)00164-8

Therefore, the ideal chromatographic separation for on-line HPLC–NMR experiments should result in small peak shapes in the order of 200 μl for all compounds under investigation.

For the separation of carotenoids, with the introduction of C_{30} stationary phases [1], their analysis using RP-materials has reached the same resolution as with the so far used normal phase chromatography. Several papers in the field of carotenoid research using C_{30} stationary phases were published, identifying the separated compounds by different spectroscopic techniques like

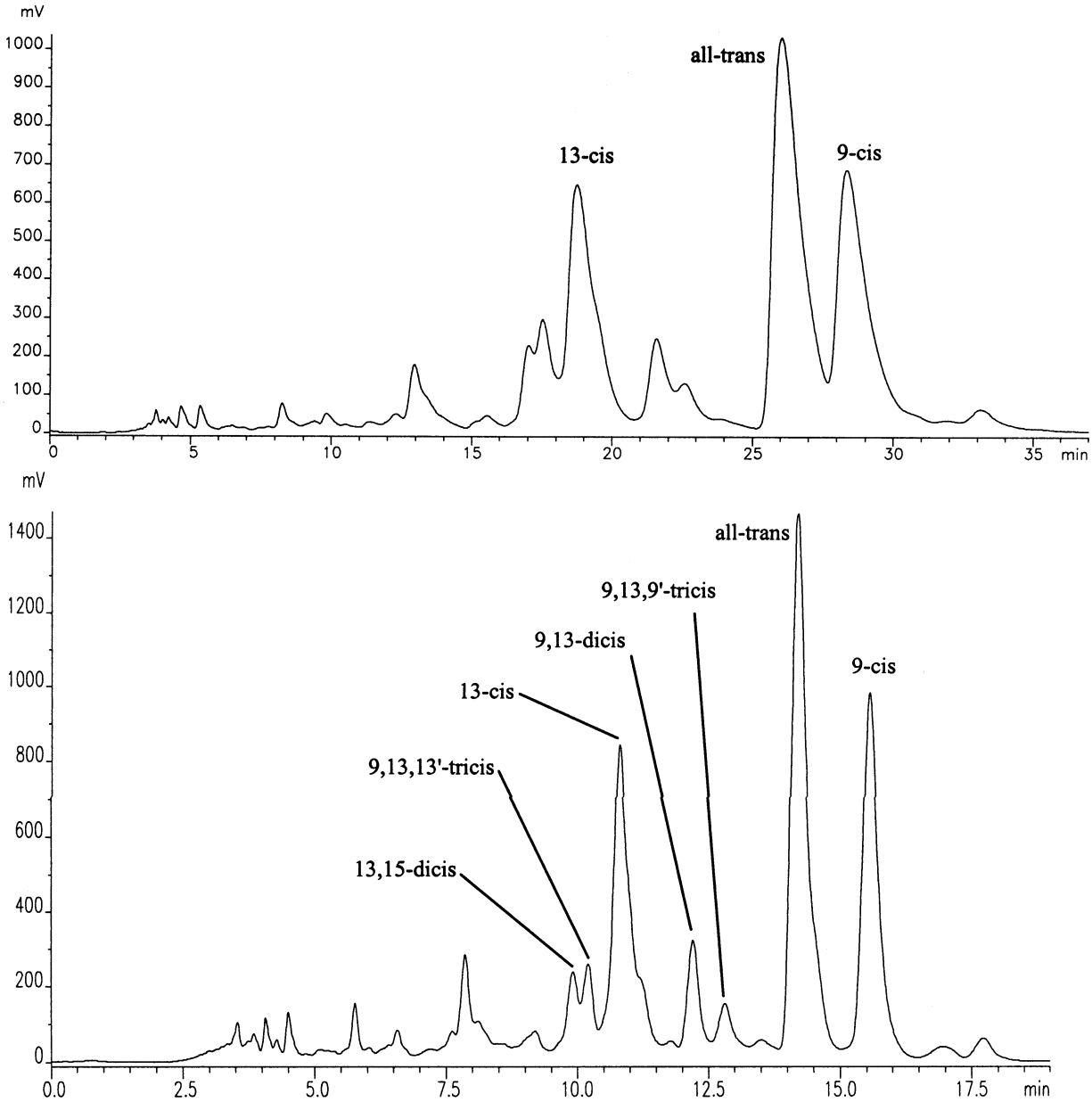

Fig. 1. Comparison of the separation of β-carotene *cis*/*trans* isomers using a 5-μm (top) and 3-μm C_{30} stationary phase (bottom).

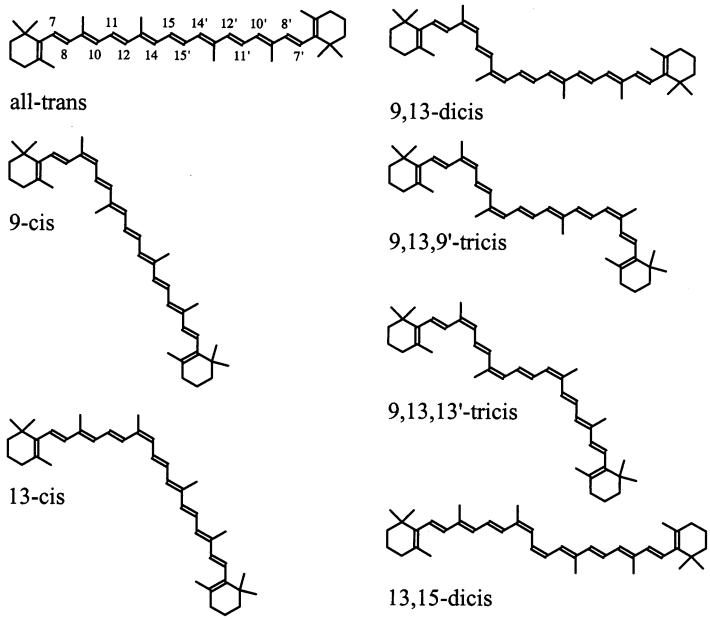

Fig. 2. Structures of the identified *cis/trans* isomers of β-carotene.

UV [2,3], MS [4,5] and NMR [6,7]. Additionally, systematic studies of the influence of temperature [8,9] and alkyl chain length [10,11] on the column performance were executed.

For the separation of carotenoid *cis/trans* isomers, C_{30} stationary phases with 5 μm diameter have been employed. We have examined the influence of particle size on the separation profile of β-carotene *cis/trans* isomers on C_{30} columns and the resulting effect on HPLC–^{1}H-NMR detection. Although 3-μm particles have been described in previous work [6], no comparison was shown between 3- and 5-μm particles. In this paper we show that for the determination and structure elucidation of minor compounds in complex mixtures the enhanced resolution is the crucial parameter.

2. Experimental

2.1. Materials

HPLC separations were carried out using acetone LiChrosolv gradient grade (Merck KGaA, Darmstadt, Germany) and deuteriumoxide 99.9% (Deutero GmbH, Herresbach, Germany). Thermally isomerized ß-carotene was provided from BASF AG (Ludwigshafen, Germany).

2.2. Columns

2.2.1. 5-μm column

A 5-μm, 200 Å YMC silica gel (YMC Europe GmbH, Schermbeck, Germany) was modified with triacontyltrichlorosilane (ABCR, Karlsruhe, Germany) in a similar fashion as described previously [1] [9]. The stationary phase was slurry packed into a 250 × 4.6-mm stainless steel column (Bischoff, Leonberg, Germany).

2.2.2. 3-μm column

A 3-μm, 200 Å silica gel (research sample, Merck KGaA, Darmstadt, Germany) was modified with triacontyltrichlorosilane (ABCR, Karlsruhe, Germany). The stationary phase was packed into a 250 × 4.6 mm stainless steel column (Bischoff, Leonberg, Germany).

2.3. HPLC–NMR coupling

Separations were performed under ambient conditions using a Merck LiChrograph L-6200A gradient pump and a Merck LiChrograph L-4000/4200 UV/Vis detector. The solvent composition was kept isocratic using acetone: D_2O, 92:8 and 93:7 for the 5- and 3-μm columns, respectively. A flow rate of 1 ml/min and a detection at 450 nm were used. For the coupling experiments, 10–20 μl of a 2% solution were injected onto the column. The amount of sample detected of each isomer

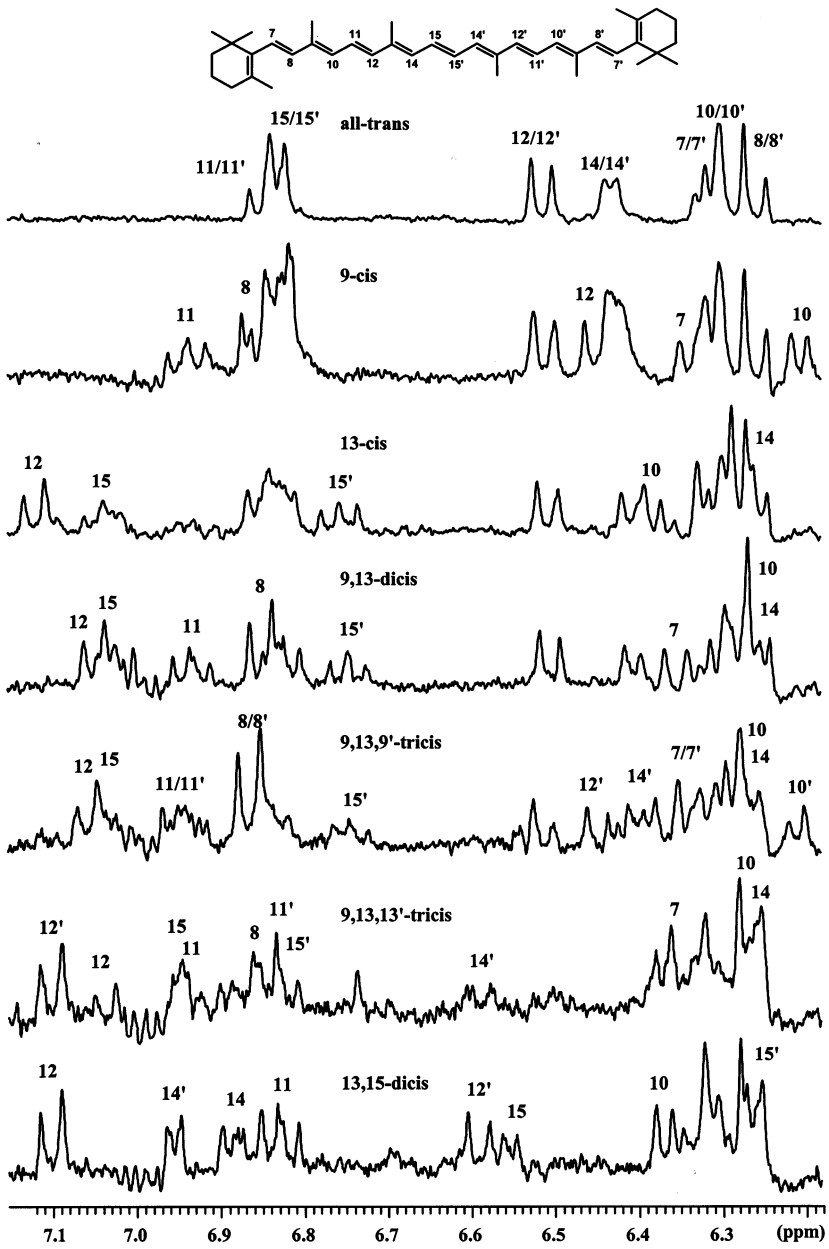

Fig. 3. Stacked-plot of the *stopped-flow* ^{1}H-NMR spectra of all β-carotene isomers.

Table 1
^{1}H-NMR chemical shift values for the β-carotene isomers determined by HPLC–NMR coupling[a]

Proton	All-*trans*	9-*cis*	13-*cis*	9,13-di*cis*	9,9′,13-tri*cis*	9,13,13′-tri*cis*	13,15-di*cis*
H-7	6.32	6.33	6.31	6.36 (+0.04)	6.36 (+0.04)	6.33	6.33
H-7′	6.32	6.32	6.31	6.32	6.36 (+0.04)	6.33	6.33
H-8	6.26	6.86 (+0.60)	6.25	6.86 (+0.60)	6.86 (+0.60)	6.84 (+0.58)	6.25
H-8′	6.26	6.25	6.25	6.26	6.86 (+0.60)	6.26	6.25
H-10	6.31	6.20 (-0 09)	6.38 (+0.07)	6.29	6.28 (-0.03)	6.26 (-0.05)	6.36 (+0.05)
H-10′	6.31	6.31	6.29	6.31	6.20 (-0.11)	6.35 (+0 04)	6.30
H-11	6.84	6.93 (+0 09)	6.84	6.94 (+0.10)	6.94 (+0.10)	6.92 (+0.08)	6.82
H-11′	6.84	6.84	6.83	6.83	6.93 (+0-09)	6.83	6.87 (+0.03)
H-12	6.51	6.45 (-0.06)	7.12 (+0.61)	7.06 (+0.55)	7.05 (+0∼54)	7.03 (+0.52)	7.10 (+0.59)
H-12′	6.51	6.51	6.50	6.51	6.44 (-0-07)	7.10 (+0.59)	6.58 (+0.07)
H-14	6.43	6.42	6.27 (-0.16)	6.27 (-0.16)	6.26 (-0.17)	6.24 (-0.19)	6.87 (+0.44)
H-14′	6.43	6.42	6.41	6.41	6.40 (-0-03)	6.58 (+0.15)	6.95 (+0.52)
H-15	6.83	6.82	7.03 (+0.20)	7.02 (+0 19)	7.01 (+0.18)	6.94 (+0.11)	6.55 (-0.28)
H-15′	6.83	6.82	6.75 (-0.08)	6.75 (-0.08)	6.74 (-0 09)	6.86 (+0.03)	6.26 (-0.57)

[a] The values in brackets denote the difference (in ppm) to the chemical shift value of the corresponding proton of all-*trans* β-carotene.

came up to about 60 μg for all-*trans*, 13-*cis*, and 9-*cis*, 24 μg of 9,13-di*cis*, and 9 μg of 13,15-di*cis*, 9,9′,13-tri*cis*, and 9,13,13′-tri*cis*.

HPLC–NMR experiments were conducted on a Bruker AMX 600 spectrometer. The chromatographic equipment and BPSU (Bruker Peak Sampling Unit) necessary for stopped-flow experiments were controlled by Chromstar software (Bruker). Detection of ^{1}H-NMR spectra was performed with an inverse probe with a detection volume of 120 μl. The deuterium signal of D_2O served as lock signal for spectrometer stability.

2.3.1. 1-D ^{1}H-NMR spectra

Solvent signal suppression of the acetone were performed using low power presaturation for 1.6 s, resulting in a receiver gain of 512. For all experiments, a time domain consisting of 32 K computer points and a spectral width of 8474 Hz were used. Two hundred and fifty six transients were coadded per FID for the all-*trans* isomer, 512 transients for the 13-*cis* and 9-*cis* isomer, and 2048 transients for all other isomers. Prior to Fourier transformation (FT), an exponential multiplication corresponding to a line broadening factor of 1 Hz was applied. For chemical shift calibration, the suppressed signal of acetone was set to 2.2 ppm.

2.3.2. 2-D ^{1}H-NMR spectra

For the 2-D ^{1}H-COSY 45 spectra, the number of experiments in the F1-dimension was set to 256 for the all-*trans*, 9-*cis*, and 13-*cis* isomer, and between 360 and 400 for the other β-carotene isomers. Sixteen scans were added for each FID for the all-*trans* isomer, 36 for 13-*cis*, 40 for 9-*cis*, 128 for 9,13-di*cis*, and 160 for all other isomers. A time domain of 2 K and sweep width of 8487.6 Hz were used. Solvent signal suppression for 1 s resulted in a receiver gain of 512. The overall experiment time amounted to about 1.5 h for the all-*trans*, 3 h for 9-*cis* and 13-*cis*, and 20 h for all the other isomers. Processing was done with XWINNMR (Bruker) on an Indy workstation (Silicon Graphics Inc.). Each FID was multiplied with a pure sine wave function before FT, and 2 K of data points have been used in both directions. After baseline correction the spectra were symmetrized about the diagonal.

3. Results and discussion

We previously reported the structure elucidation of β-carotene isomers by HPLC–NMR coupling using a 5-μm C_{30} bonded phase [7]. In this work, the structure of five isomers had been deter-

mined, from the three most abundant isomers the structure could be confirmed by 2-D NMR spectroscopy. The corresponding chromatogram is shown in the upper part of Fig. 1. Although the selectivity towards the *cis*/*trans* isomers of β-carotene isomers is good, the overall peak shape

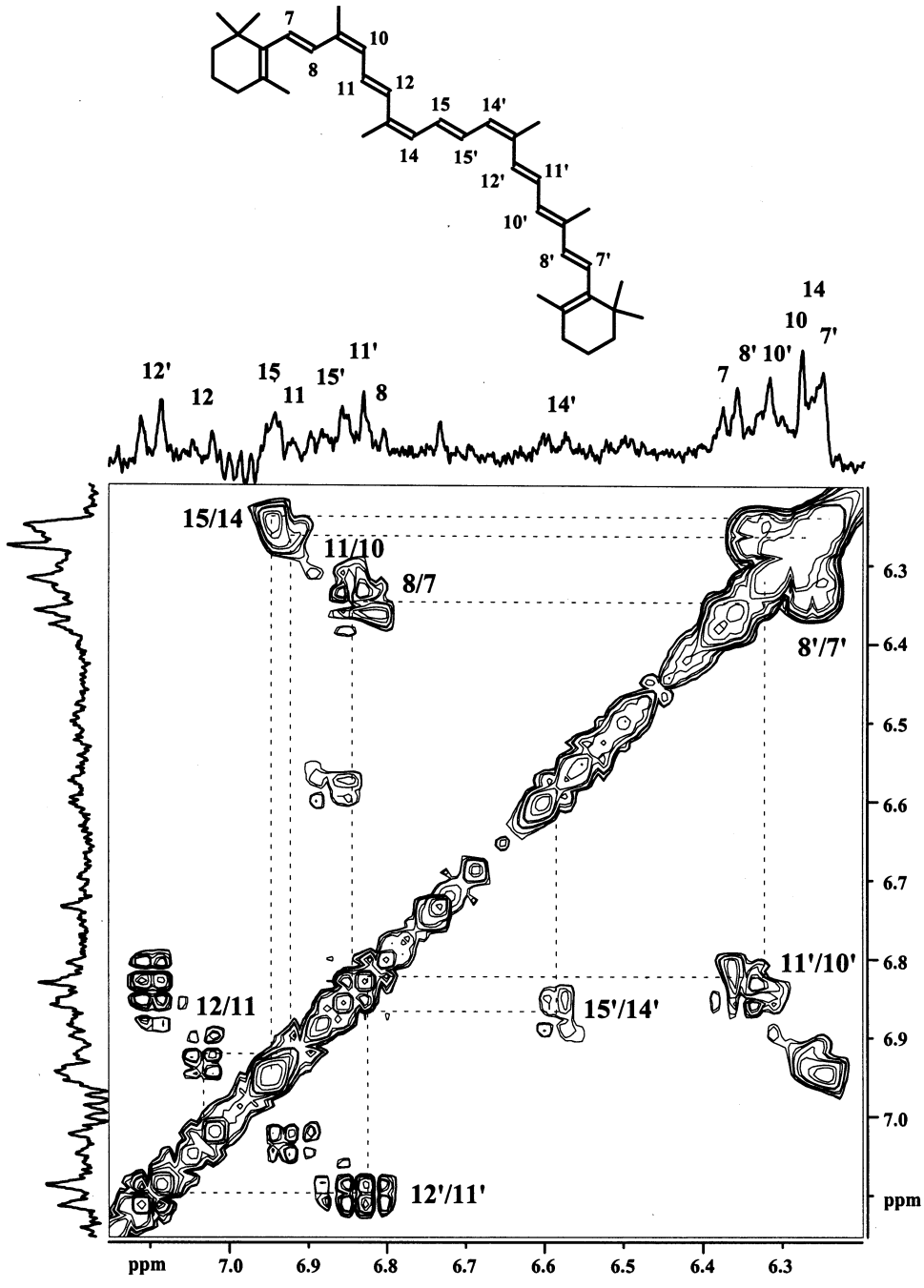

Fig. 4. Olefinic part of a COSY 45 spectrum of 9,13,13′-tri*cis* β-carotene.

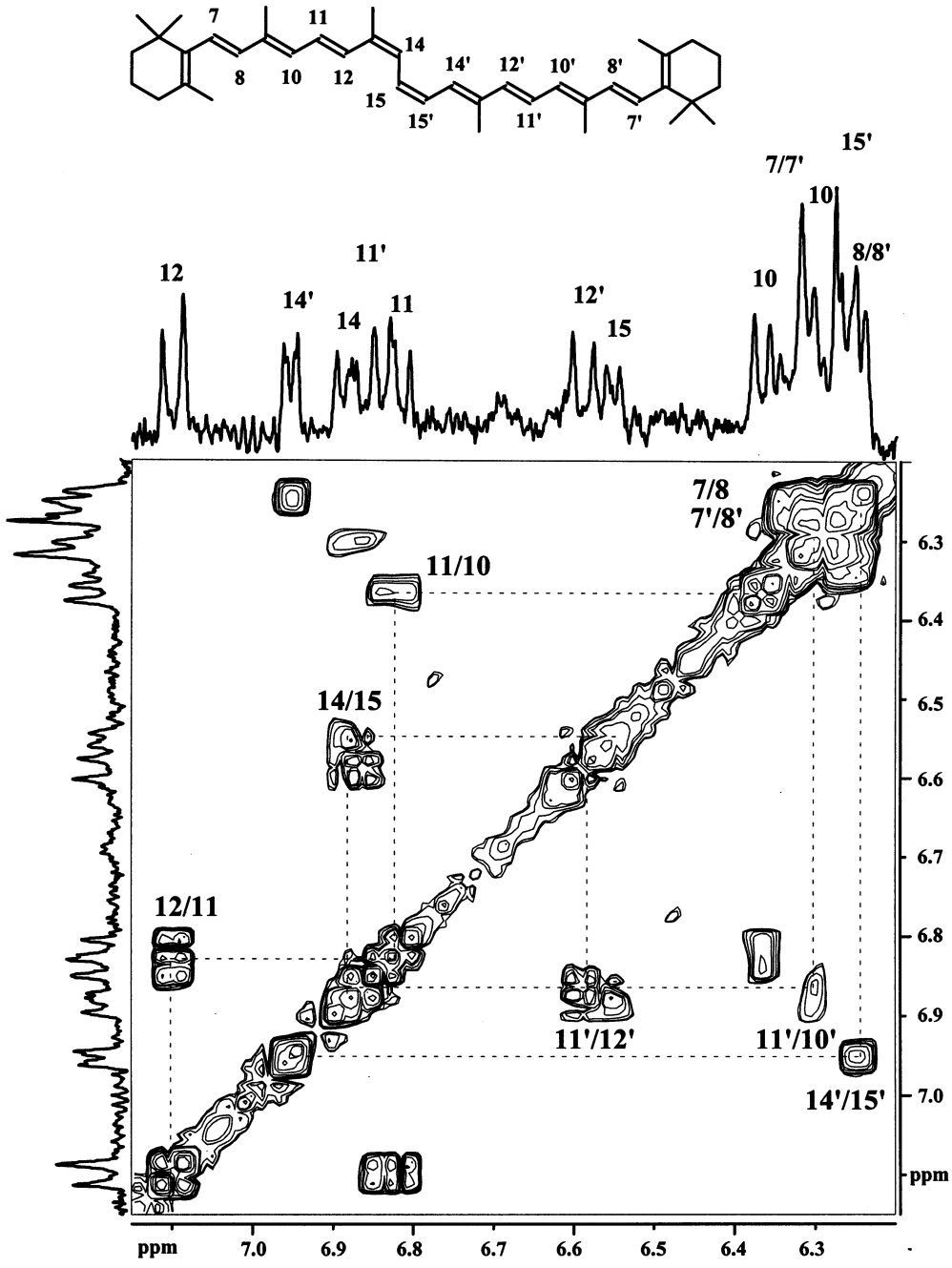

Fig. 5. Olefinic part of a COSY 45 spectrum of 13,15-di*cis* β-carotene.

is quite broad. A significant degree of column overloading was necessary for these HPLC–NMR experiments. Due to the broad peaks the highest amount of sample to be put onto the column soon reached its limit and peak overlap occurred. Therefore, the concentration of the sin-

gle isomers was limited, so that only the acquisition of 2-D COSY spectra for the three most abundant isomers was possible.

The lower chromatogram of Fig. 1 shows the separation of the same sample mixture with similar conditions, but with a 3-μm C_{30} stationary phase. The solvent composition was altered slightly resulting in shorter retention times. It can be seen that the selectivity of the two columns is similar, whereas the 3-μm column exhibits a much better line shape. This leads to a better resolution of the isomers and consequently to a better separation. As an example, the all-*trans* and 9-*cis* isomers are fully baseline-separated, the two major compounds eluting before 13-*cis* are resolved, and several minor compounds can additionally be detected. The structures of all seven identified isomers of β-carotene are displayed in Fig. 2 while the corresponding ^{1}H-NMR spectra of all identified β-carotene isomers are shown as stacked-plot in Fig. 3. Here a complete assignment of all olefinic protons is given for the all-*trans* isomer. For all *cis*-isomers, only those resonances are labeled which appear due to the new introduced *cis*-bond. For comparison, the chemical shift values of all determined structures and their shift relative to the all-*trans* values are listed in Table 1. A comparison with those data recorded in chloroform shows large differences for the absolute chemical shift values. The relative shift between all-*trans* and the *cis*-isomers on the other hand are in accordance with the literature [12].

The change in separation efficiency has dramatic effects for *on-line* coupling experiments like HPLC–NMR coupling. Due to the better lineshape, with a given concentration injected onto the column, the concentration of every isomer at its peak maximum is much higher with the 3-μm than with the 5-μm column. This is due to the smaller elution volume of each isomer. In the NMR experiments, a 120-μl detection cell is generally used. This volume is normally smaller than the peak volume of the desired compound. Therefore the amount detected by the NMR spectrometer is not determined by the overall amount of sample in one peak but by the concentration of sample at the peak maximum. As an example, the 2-D NMR spectra, the COSY 45 spectrum of 9,13,13′-tri*cis* β-carotene and 13,15-di*cis* β-carotene are shown in Fig. 4 and Fig. 5, respectively. Due to the improved peak shape of the separation, the acquisition of 2-D NMR spectra for an unambiguous structural assignment was possible even for these minor compounds in the isomer mixture.

By taking a look at the elution order of these various isomers, an interesting observation can be made. Taking all-*trans* β-carotene as reference, the 13-*cis* isomer is eluting earlier while the 9-*cis* isomer exhibits a stronger retention. For the di*cis* and tri*cis* isomers this effect seems to be additive, i.e. 9,13-di*cis* β-carotene elutes between 9-*cis* and 13-*cis*. Also, 9,13,13′-tri*cis* β-carotene elutes earlier than 13-*cis*, while 9,9′,13-tri*cis* β-carotene elutes later than 9,13-di*cis*. Further mechanistic studies about the retention behavior and surface structure of C_{30} interphases might give further explanations for this unique feature [13].

Acknowledgements

The authors thank Tanja Lacker and Markus Dachtler for critical comments and for reading the manuscript.

References

[1] L.C. Sander, K.E. Sharpless, N.E. Craft, S.A. Wise, Anal. Chem. 66 (1994) 1667–1674.
[2] C. Emenhiser, L.C. Sander, S.J. Schwartz, J. Chromatogr. A 707 (1995) 205–216.
[3] C. Emenhiser, N. Simunovic, L.C. Sander, S.J. Schwartz, J. Agric. Food Chem. 44 (1996) 3887–3893.
[4] R.B. van Breemen, C.-R. Huang, Y. Tan, L.C. Sander, A.B. Schilling, J. Mass Spectrom. 33 (1996) 975–981.
[5] R.B. van Breemen, Anal. Chem. 68 (1996) 229A–304A.
[6] C. Emenhiser, G. Englert, L.C. Sander, B. Ludwig, S.J. Schwartz, J. Chromatogr. A 719 (1996) 333–343.
[7] S. Strohschein, M. Pursch, H. Händel, K. Albert, Fresenius, J. Anal. Chem. 357 (1997) 498–502.
[8] C.M. Bell, L.C. Sander, S.A. Wise, J. Chromatogr. A 757 (1997) 29–39.
[9] M. Pursch, S. Strohschein, H. Händel, K. Albert, Anal. Chem. 68 (1996) 383–393.

[10] C.M. Bell, L.C. Sander, J.C. Fetzer, S.A. Wise, J. Chromatogr. A 753 (1996) 37–45.
[11] S. Strohschein, G. Schlotterbeck, J. Richter, M. Pursch, L.-H. Tseng, H. Händel, K. Albert, J. Chromatogr. A 765 (1997) 207–214.
[12] ,Spectroscopy,G. Brotton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, vol. 1B, Birkhäuser Verlag, Basel, 1995.
[13] K. Albert, T. Lacker, M. Raitza, M. Pursch, H.-J. Egelhaaf, D. Oelkrug, Angew. Chem. Int. Ed. Engl. 37 (1998) 778–780.