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Comparison of the separation of *cis/trans* isomers of tretinoin with different stationary phases by liquid chromatography–nuclear magnetic resonance coupling

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Abstract

The comparison of two different types of RP stationary phases in their ability to separate *cis/trans* isomers of retinoic acid (tretinoin) has been investigated by LC–NMR coupling. Only by recording of ^{1}H NMR spectra the structural identification of the separated compounds was possible, since their absorption coefficients are very similar and their mass is identical, and therefore identification by UV–Vis is not unambiguous and identification with LC–MS fails due to identical fragmentation patterns. A commonly used C_{18} phase and a recently developed C_{30} phase have been used for the separation of a mixture of thermal isomerized retinoic acids. Three isomers could be separated and identified with the separation on a C_{18} column, whereas five *cis/trans* isomers could be identified by the use of a C_{30} column. It could be shown that even under optimized chromatographic conditions the separation efficiency of the two phases varies and that these differences are probably due to differences in the alkyl chain organization of the stationary phases.

Keywords: Stationary phases, LC; Nuclear magnetic resonance spectroscopy; Retinoic acid; Tretinoin; Vitamins

1. Introduction

Retinoic acid and its derivatives (e.g., retinol) play an important role in the metabolism of animals and humans, i.e., they are involved in the regulation of cell proliferation, vision, reproduction, and differentiation. Their role in the embryonic metabolism, especially as a growth factor and as a chemotherapeutic agent is under investigation [1–4]. They are also used as food additives and in pharmaceutical products. The biological activity of the all-*trans* and the various *cis* isomers varies [5], therefore a separation and identification of these isomers is important.

For the last years, C_{18} columns have been the preferred choice in RP (reversed-phase) chromatography. These columns give good results for the separation of various compounds, but fail in an optimal separation of carotenoids [6]. Therefore, a recently developed C_{30} phase has been introduced which shows a much better selectivity towards *cis/trans* isomers of carotenoids [7–9]. We have tested the selectivity and separation efficiency of these columns for the related compound retinoic acid and its *cis/trans* isomers.

For *cis/trans* isomers in general, the identification

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of the different compounds via UV–Vis detection is not applicable, since all isomers have a similar absorption coefficient so that exact structure determination is not possible. HPLC–MS coupling is also not practicable because *cis/trans* isomers all have the same mass, and yield identical fragmentation patterns [10]. Therefore no structural information to distinguish between the isomers is gained from MS spectra. So the method of choice for structural elucidation of *cis/trans* isomers is ^{1}H NMR spectroscopy [11]. On-line coupling of HPLC and NMR has many advantages to conventional off-line separation [12]. The off-line technique includes collecting of the fractions, followed by working up of the samples for redissolving in deuterated solvents, and recording of NMR spectra. On-line experiments are less time consuming due to subsequent separation and ^{1}H NMR detection in a closed system. Secondly, the *cis/trans* isomers of tretinoin are unstable and are readily isomerized by light, heat or chemical reactions. So in the off-line experiment, reisomerization of the fractionated sample may occur during sample preparation for the NMR experiment. In the HPLC–NMR experiment, separation and detection are performed in a closed loop. Therefore, isomerization caused by light, or decomposition caused by contact with oxygen are not possible.

2. Experimental

2.1. Materials

For HPLC separations, acetonitrile (ACN), methanol (both LiChrosolv gradient grade, Merck, Darmstadt, Germany) and [$^2H_2$]water 99.9% (Deutero, Herresbach, Germany) were used. Isomerized tretinoin (0.1% solution in tetrahydrofuran–ACN, 9:1, 1 h irradiated) was provided by BASF (Ludwigshafen, Germany).

2.2. Chromatography

Separations were carried out under ambient conditions using a Merck Lichrograph L-6200A gradient pump and a Merck Lichrograph L-4000/4200 UV–Vis detector. For all experiments, a flow-rate of 1 ml/min was used and the separation was monitored at 353 nm.

2.2.1. C_{18} column

The eluent was an isocratic mixture of acetonitrile–[$^2H_2$]water (80:20). Chromatography was performed on a LiChrospher Select B column (250 mm×4.6 mm, Bischoff, Leonberg, Germany). 40 μl of sample solution were injected onto the column.

2.2.2. C_{30} column

The eluent was an isocratic mixture of methanol–[$^2H_2$]water (73:27), Chromatography was done on a YMC C_{30} carotenoid column (250 mm×4.6 mm, YMC Europe, Schermbeck, Germany). 100 μl of sample solution were injected onto the column.

2.3. HPLC–NMR coupling

Experiments were conducted by use of a Bruker ARX 400 (C_{18}) and AMX 600 (C_{30}) spectrometer (Bruker, Rheinstetten, Germany). The chromatographic equipment and BPSU (Bruker Peak Sampling Unit) used for the stopped-flow experiments were controlled by Chromstar software (Bruker). All ^{1}H NMR spectra were recorded in the stopped-flow mode using an LC inverse NMR probe with a detection volume of 120 μl. Solvent signal suppression was achieved by low power presaturation at the corresponding frequency of the solvent signals prior to the start of the acquisition. All NMR data were processed with 1D WINNMR (Bruker).

2.3.1. C_{18} column

2 K transients were co-added per FID (free induction decay) with a time domain of 32 K and a sweep width of 6024 Hz. For all spectra, zero filling up to 64 K data points and an exponential multiplication of the FID with a line broadening of 0.3 Hz have been applied prior to Fourier transformation.

2.3.2. C_{30} column

1 K transients were co-added per FID with a time domain of 16 K and a sweep width of 9615 Hz. For all spectra, zero filling up to 32 K data points and an exponential multiplication of the FID with a line

broadening of 0.3 Hz have been applied prior to Fourier transformation.

3. Results

A comparison of the two chromatograms from the separation of *cis/trans* isomers of retinoic acid on a C_{18} column and a C_{30} column (Fig. 1and Fig. 2, respectively) shows that in the former experiment only three peaks of *cis/trans* isomers are visible, whereas in the latter experiment five peaks are separated. This could lead to the conclusion, that on the C_{30} column, five isomers are eluting subsequently, while on the C_{18} column two peaks must consist of two coeluting isomers. The hyphenation of chromatography with ^{1}H NMR spectroscopy in contrast reveals that on the C_{18} column three isomers are eluting in high concentrations, while the other existing isomers are coeluting in low concentrations as broad peaks and are not detected either NMR spectroscopically or by UV–Vis. For the chromatography on the C_{30} column, the five peaks labeled in Fig. 2 correspond to six compounds, two of them coeluting.

The stopped-flow ^{1}H NMR spectra shown in Fig. 3 and Fig. 4 correspond to the separation on a C_{18} column and on a C_{30} column, respectively. Only the olefinic part of the spectra is displayed. The order of the spectra from bottom to top reflects the elution order of both chromatographic separations. The structures of all identified compounds, i.e., *cis/trans* isomers and one oxidized component, are depicted in Fig. 5.

In the experiment using a C_{18} column for separation, stopped-flow ^{1}H NMR spectra of the three dominant peaks of the chromatographic separation in Fig. 1 have been recorded. As shown in Fig. 3, by means of HPLC–NMR coupling these three chromatographic peaks could be identified as 9-*cis*, 13-*cis*, and all-*trans* tretinoin.

In the experiment using a C_{30} column for separation, stopped-flow ^{1}H NMR spectra have been recorded for all *cis/trans* isomers of tretinoin as well

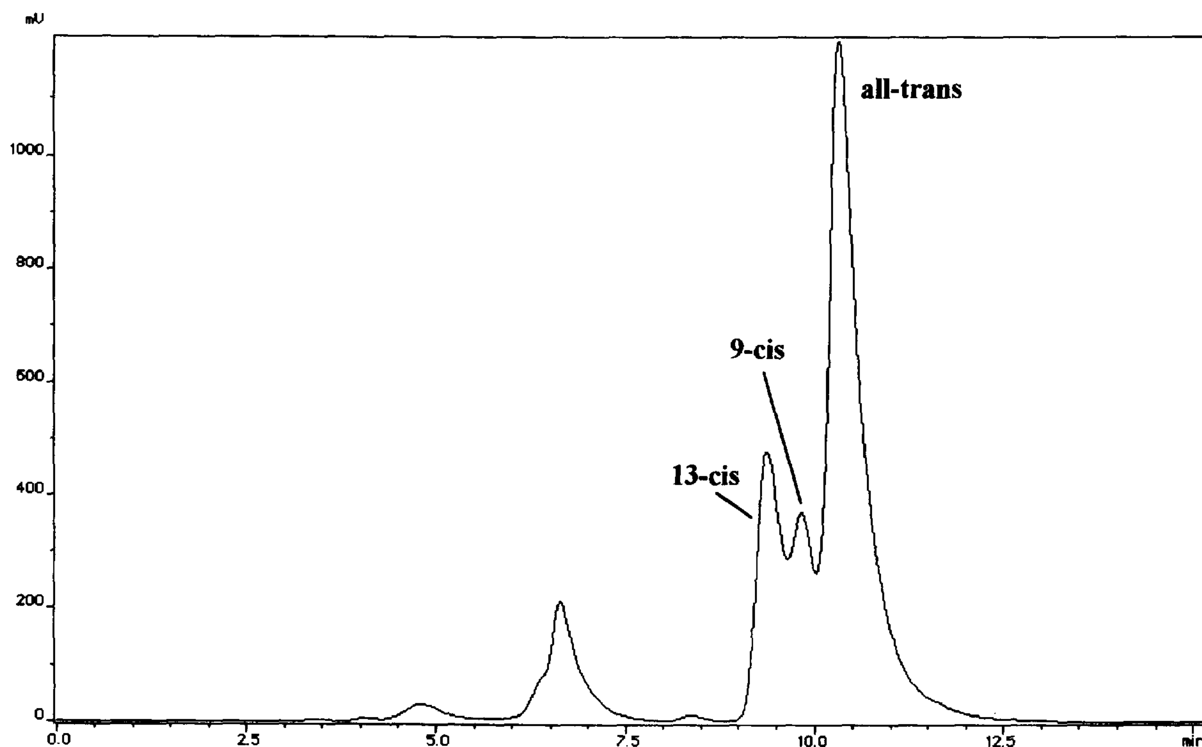

Fig. 1. UV chromatogram of the separation of tretinoin *cis/trans* isomers on a C_{18} column.

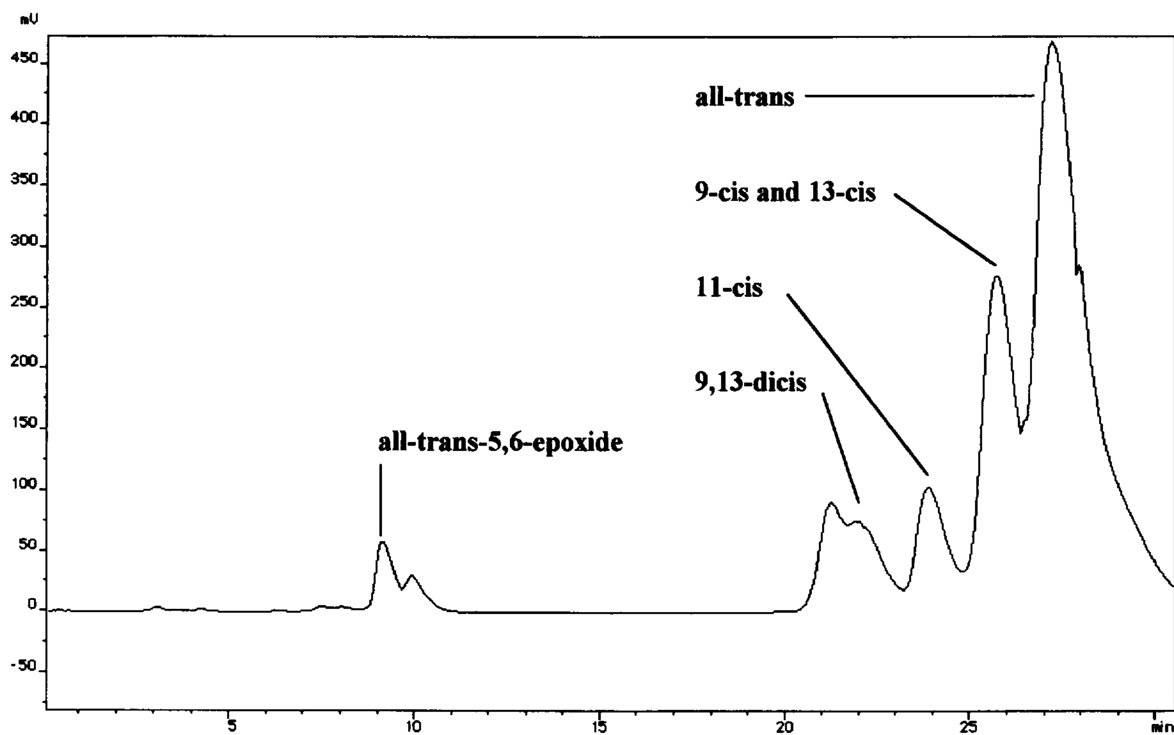

Fig. 2. UV chromatogram of the separation of tretinoin *cis/trans* isomers on a C_{30} column.

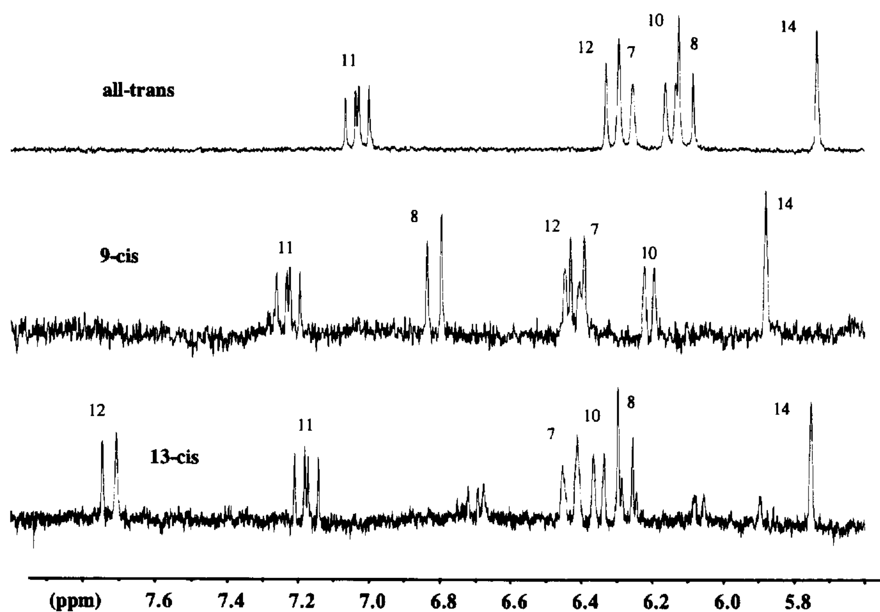

Fig. 3. Stacked plot of the 400 MHz 1D ^{1}H NMR spectra of *cis/trans* isomers of tretinoin separated on a C_{18} column.

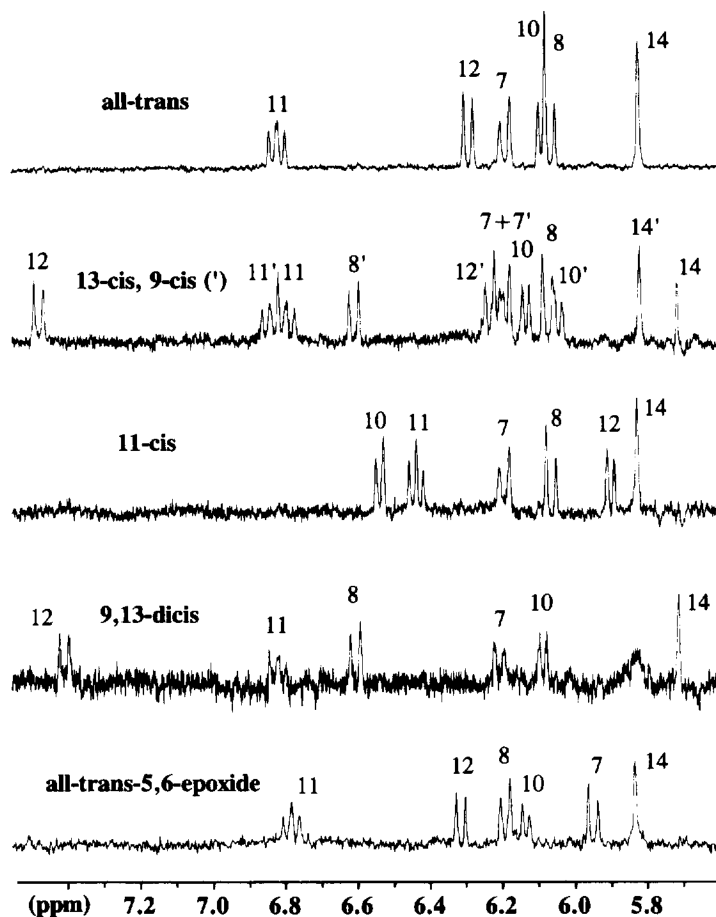

Fig. 4. Stacked plot of the 600 MHz 1D ^{1}H NMR spectra of *cis/trans* isomers of tretinoin separated on a C_{30} column.

as for the dominant peak of the earlier eluting components in the chromatographic run. The structure of the compound corresponding to the retention time of 21 min is still under discussion. As indicated in Fig. 4, the peak corresponding to an elution time of 9.1 min could be identified as all-*trans*-5,6-epoxytretinoin. The structural assignment for the *cis/trans* isomers are as follows in order of elution time: 9,13-di*cis*, 11-*cis*, 9-*cis* and 13-*cis* coeluting, and all-*trans* tretinoin. The chemical shift of the olefinic protons of all solutes measured under the given conditions are shown in Table 1 and the corresponding coupling constants are displayed in Table 2.

RP separations are often carried out with acetonitrile–water as mobile phase. For the detection of oxidized compounds of tretinoin, such as epoxytretinoin, this solvent mixture is not applicable, since the resonance of the methyl group of acetonitrile in the ^{1}H NMR spectrum interferes with the signals of the aliphatic protons of the solute. For this reason, the separation on the C_{30} column was performed with methanol–water as mobile phase. So, it was possible to detect the resonance shift of the signal of the methyl groups attached at the carbon atom C-1. In the ^{1}H NMR spectrum of all-*trans* tretinoin, the two methyl groups have an identical environment and therefore give only one resonance signal at 0.98 ppm. Due to the epoxide bridge between the carbon atoms C-5 and C-6, these methyl groups get a different chemical environment, which leads to two distinguishable signals at 0.88 ppm and 1.07 ppm.

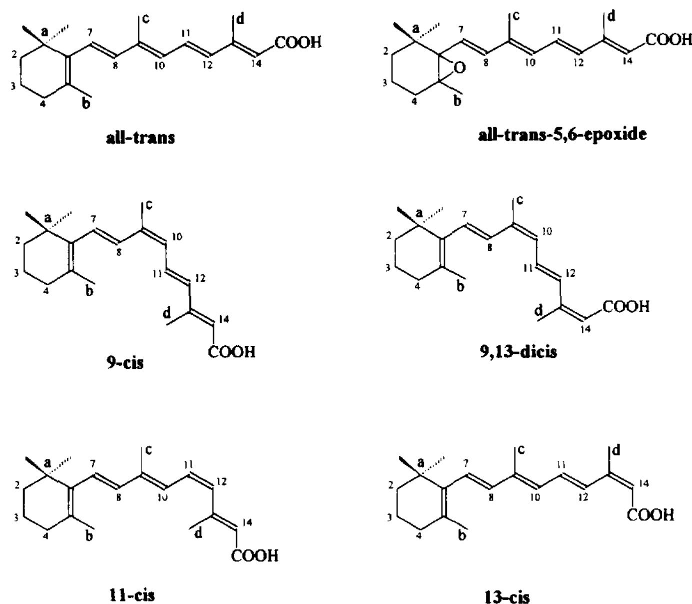

Fig. 5. Structures of the identified *cis/trans* isomers of tretinoin and all-*trans*-5,6-epoxytretinoin.

Table 1
Chemical shift values in ppm of the *cis/trans* isomers of tretinoin and all-*trans*-5,6-epoxytretinoin

	H-7	H-8	H-10	H-11	H-12	H-14
all-*trans*-5,6-epoxide	5.95	6.19	6.14	6.79	6.32	5.84
9,13-di*cis*	6.19	6.61	6.08	6.82	7.41	5.71
11-*cis*	6.19	6.06	6.46	6.39	5.93	5.89
9-*cis*	6.23	6.61	6.04	6.84	6.23	5.82
13-*cis*	6.19	6.07	6.13	6.79	7.48	5.72
all-*trans*	6.19	6.06	6.09	6.82	6.29	5.82

The epoxide bridge also has an effect on the olefinic protons H7 and H8. The ^{1}H NMR signals of these protons reverse their position compared with the parent compound all-*trans* tretinoin. The aliphatic and olefinic part of the ^{1}H NMR spectrum of all-*trans*-5,6-epoxytretinoin are shown in Fig. 6.

Table 2
Coupling constants in Hertz of the *cis/trans* isomers of tretinoin and all-*trans*-5,6-epoxytretinoin

	H-7/H-8	H-10/H-11	H-11/H-12
all-*trans*-5,6-epoxide	15.0	11.3	15.8
9,13-di*cis*	16.0	11.5	14.7
11-*cis*	16.4	11.8	12.3
9-*cis*	16.0	11.3	14.8
13-*cis*	16.5	12.3	15.3
all-*trans*	16.0	11.4	15.0

4. Discussion

Optimization of the chromatographic conditions for the C_{18} column has led to three separated peaks, whereas with the use of a C_{30} column five peaks could be separated. The comparison of the two chromatographic runs clearly shows that for a peak assignment it is not sufficient to rely upon retention times. The on-line coupling of HPLC with NMR spectroscopy revealed that in the first separation (C_{18}), the main peaks in the UV chromatogram belong to isolated compounds, whereas in the second

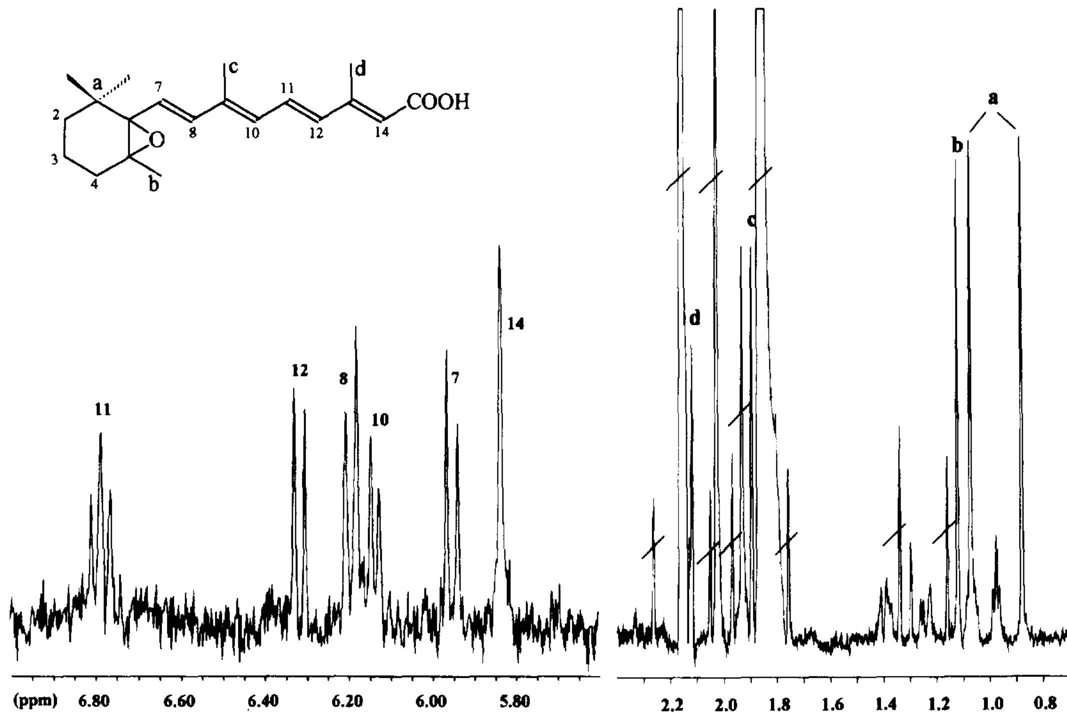

Fig. 6. Aliphatic and olefinic part of the 1D ^{1}H NMR spectrum of all-*trans*-5,6-epoxytretinoin. The marked signals in the aliphatic part do not belong to the substance but are impurities from the solvent.

separation (C_{30}), more isomers are separated but even so two isomers do coelute. By judging the chromatograms themselves, one would have predicted the contrary, i.e., peaks that were supposedly coeluting on the C_{18} column were separated on the C_{30} column.

The differences in chromatographic elution order and separation efficiency of the two investigated columns can be due to variation of the mobile phase or due to change of the stationary phase. The separation efficiency of retinyl acetate isomers on a C_{30} column has been recently investigated [13]. Additionally, it was shown that C_{30} columns have a better ability to distinguish between *cis/trans* isomers of carotenoids than C_{18} columns. This capability depends on the order of the alkyl chains of the stationary phase, which is higher for C_{30} columns, and is increasing with subambient temperatures. The changing of solvents from one separation to the other of course has an effect on the separation itself. But the differences in alkyl chain organization of both stationary phases are probably mainly responsible for the observed differences in the chromatographic performances of the C_{18} and C_{30} columns.

Acknowledgments

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