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High-performance liquid chromatographic analysis of retinal and retinol isomers

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Abstract

Normal-phase chromatograms of retinal- and retinol isomers using various mobile phases are presented, showing that in *n*-hexane–*tert.*-butyl methyl ether and also in *n*-heptane–*tert.*-butyl methyl ether the elution order for retinol is 13-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-retinol, whereas in *n*-hexane–1,4-dioxane 11-*cis*- elutes before 13-*cis*-retinol. Assignment of these main retinal and retinol peaks was performed using pure crystals of isomers, measurement of absorbance spectra and analysis of NMR spectra. The new mobile phase *n*-heptane–*tert.*-butyl methyl ether allows best baseline separation of the commonly occurring isomeric forms in reasonably short analysis time. In addition, eight di-*cis*- and tri-*cis*-retinol isomers were tentatively identified. The chromatograms show that former identifications in the literature are inconsistent or wrong.

Keywords: Mobile phase composition; Retinol; Retinal; Vitamin A

1. Introduction

The investigation of the transformation and regeneration steps of rhodopsin and its retinoid chromophore during the visual cycle [1–3], the analysis of the vitamin A content in nutritional science [4], in samples of liver [5] or corpus luteum [6], depends to a great extent on the exact identification of the various isomeric forms of retinal and retinol. Only a few of these isomers (all-*trans*-, 13-*cis*-, 9-*cis*-) are relatively stable, which means that results are dependent on the solvents used, the kind of extraction from the tissues, the temperature and speed of the measurement, ambient light, and sample storage conditions, just to name a few parameters. High-performance liquid chromatography (HPLC) is a common method for the separation of the different conformations of retinal and retinol, sometimes in the reversed-phase mode [7] but mainly in the normal-phase mode [1,4,8,9]. For identification usually reference is made to a few chromatograms reported in the literature, since own identification of the isomers is complicated, because standards or authentic material are mostly not available. In addition, a diode-array detector e.g., which would aid in identification by allowing the immediate measurement of the absorption spectra of unstable or small products, does not belong to the standard equipment of HPLC setups in a laboratory, and ^{13}C or ^{1}H NMR spectroscopy is commonly not available and requires relatively large samples, in the range of milligrammes.

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In order to get reproducible results, the isocratic mode is to be preferred, when all the elution parameters have been established. A very reliable identification of retinal conformations was shown by Sperling et al. [10] and Bruening et al. [11]. They described 19 retinal isomers, however, using the uncommon eluent mixture freon 113–methyl *tert.*-butyl ether (97:3, v/v). But there is no standard chromatogram for all the different retinol isomers. The published chromatograms of the main conformations, mostly using *n*-hexane–dioxane, are inconsistent and even wrong [1,8,12–14]. This has consequences for all published and further experiments and conclusions referring to these papers [15].

In the following, therefore, chromatograms are presented of retinal isomers and of retinol conformations, derived from the retinals, both at different eluent compositions. We used *n*-hexane–*tert.*-butyl methyl ether and *n*-heptane–*tert.*-butyl methyl ether. For comparison we repeated also measurements of other laboratories with their parameters and reconsidered a still widely used, but toxic, mobile phase which contains the supposedly carcinogenic 1,4-dioxane [16] and the toxic *n*-hexane [17].

The identifications and assignment of the various conformations were performed with authentic material and by cochromatography applying mixtures of retinal and retinol isomers, respectively; they were verified by measurement of their UV-absorbance spectra, their NMR spectra, by data from original crystals and by comparison with chromatograms of Sperling [10 and unpublished] and Nakanishi's laboratory (e.g. Bruening et al. [11]).

2. Experimental

2.1. Instrumentation

Analysis of the retinal and retinol isomers was performed with a HPLC System 400 (Kontron Instruments) consisting of a HPLC pump 420 and a HPLC gradient former GF 425. The eluents were pumped through a degasser (Erma ERC-3312) and mixed on the low-pressure side. During this study we used only the isocratic mode. A Kontron UV detector 432 with variable wavelength transmitted the absorption values (photometric range usually 0.01 AUFS) to the Kontron 450-MT data system (Softron). For measurement of the retinal isomers the absorption maximum of all-*trans*-retinal in *n*-hexane at 371.34 nm was chosen, and 325 nm, the maximum of all-*trans*-retinol in *n*-hexane, was selected for recording the retinol isomers. If the relative and absolute amount of the various isomers had to be evaluated, we used the correction factors shown in Table 1. The corresponding absorbance maxima were derived from crystalline material, dissolved in *n*-hexane (Sperling, unpublished).

The chromatographic system was equipped with a Rheodyne injection valve (Model 7125) with a sample volume of 20 μl. Injections were made manually with a 5-, 10- or 25-μl Hamilton syringe. Adsorption chromatography was accomplished with a DuPont column Zorbax SIL (25 cm × 4.6 mm I.D.) packed with Si60 porous spherical particles of 5–6 μm in diameter and occasionally, for comparison, with a Merck column LiChrospher (25 cm × 4 mm I.D.) packed

Table 1
Correction factors accounting for the different absorbance maxima of retinal and retinol isomers in *n*-hexane, using the all-*trans*-form as reference

	At λ = 371 nm		At λ = 325 nm
all-*trans*-Retinal	1	all-*trans*-Retinol	1
13-*cis*-Retinal	1.31	13-*cis*-Retinol	1.12
11-*cis*-Retinal	1.90	11-*cis*-Retinol	1.53
9-*cis*-Retinal	1.34	9-*cis*-Retinol	1.28

with Si60 5-μm silica. All chromatograms were obtained at 25°C and under dim red light.

2.2. Reagents

1,4-Dioxane Chromasol was purchased from Riedel–de Haën, *n*-hexane, *n*-heptane, *tert.*-butyl methyl ether (*t*BME) from Merck (all LiChrosolv). We preferred *t*BME, because it has a lower vapour pressure (417 mbar) compared to diethyl ether (587 mbar), which is commonly used but has the disadvantage of generating gas bubbles in the solvent feed pipes on the low-pressure side. All other solvents used, such as light petroleum (GR boiling range 40–60°C), ethanol, 2,2,2-trifluoroethanol, and the various chemicals necessary for preparation of the isomers were of analytical grade (Merck).

2.3. Samples

Retinal isomers were prepared from the all-*trans*-isomer (Aldrich) according to Gilardi et al. [18], in the beginning partly in the laboratory of W. Sperling (IBI, Forschungszentrum Jülich, Germany), where the detailed data for pure material, e.g. data of crystal structures, and the experience in crystallization from authentic seed crystals (13-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-retinal) were available. The retinal isomers were chromatographed by LC and HPLC and analyzed according to Sperling et al. [10]. All the different retinal isomers, which arise from isomerizing an all-*trans*-retinal sample with white light, were collected and samples of each fraction reduced to the corresponding retinol isomeric form by sodiumborohydride [19]. Aliquots were then used to create standard runs, to calibrate, identify and assign the retinal and retinol chromatograms via cochromatography, respectively.

In order to get additional different mixtures of retinal isomers for test runs, we illuminated all-*trans*-retinal in various solvents under a 75-W bulb (Osram) of a simple desk lamp in quartz cuvettes on ice. The amount, kind and number of isomers depended on the bleaching time, ranging from 20, 40, 60, 120 to 180 min, and were highest in 2,2,2-trifluoroethanol at 120 min. We dried the mixtures of isomers under a gentle stream of nitrogen gas and kept them in the freezer until use. The samples were then diluted in *n*-hexane, to get a reasonable signal-to-noise ratio for 5, 10 or 20 μl per injection.

Storage of samples was best at −70°C after drying in a micro test tube (Safe-Lock 0030120.094 from Eppendorf). For a few days they also could be kept in a freezer at −25°C. The following retinal isomers, however, have a very short life time (hours and less) and presumably decay to other di- and tri-*cis*-isomers (in parenthesis): 9,11,13-tri-*cis* (9,13-di-*cis*), 7,11-di-*cis* (9,11-di-*cis*, 7,13-di-*cis* and 13-*cis*), 7-*cis* (all-*trans*), the same being likely for some retinol isomers, e.g. 7,11-di-*cis* (9,11,13-tri-*cis*).

2.4. Identification

The assignment and purity of the retinal and retinol isomers was also determined by measuring the absorbance spectra of the various samples with a double-beam spectrophotometer (Hitachi 557-0009) from 500 to 190 nm, by comparison of retention times with chromatograms of our authentic samples and with the literature, if available. Additionally, the main peaks (13-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-retinal) were verified by ^{13}C and ^{1}H NMR spectroscopy (Bruker AM 400 WB [2,20]), both from standard material and from collected fractions.

3. Results

3.1. Retinal isomers

n-Hexane–tert.-butyl methyl ether

All-*trans*-retinal was dissolved in 2,2,2-trifluoroethanol (1 mg in 1 ml solvent) and exposed on ice for 60 min to the white light of a desk lamp (Osram 75-W bulb). Dried under nitrogen gas and redissolved in *n*-hexane, up to 19 retinal isomers could get separated in an aliquot of this irradiated sample. Fig. 1 shows a chromatogram with the mobile phase *n*-hexane–*tert.*-butyl methyl ether (*t*BME) (97:3, v/v), which matches closely a chromatogram of this sample in the

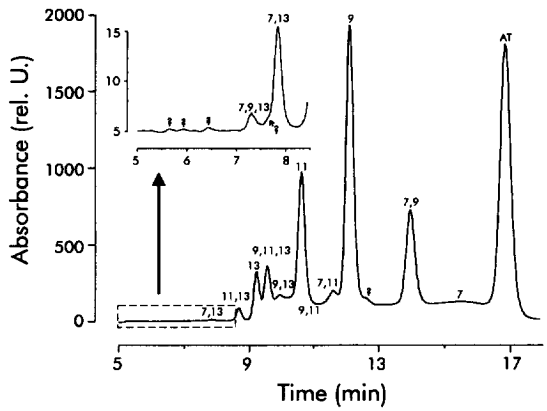

Fig. 1. Chromatogram of retinal isomers with the mobile phase *n*-hexane–*tert.*-butyl methyl ether(*t*BME) (97:3, v/v). The all-*trans*-retinal sample was isomerized with white light in 2,2,2-trifluoroethanol, dried under nitrogen gas and redissolved in *n*-hexane. Flow-rate 2 ml/min, 57 bar, measuring wavelength 371 nm. The elution order is given in Table 2, peak detection 0.1 ng, DuPont column Zorbax SIL.

commonly used eluent system *n*-hexane–diethyl ether. The elution order is given in Table 2.

In 2,2,2-trifluoroethanol preferentially the 11-*cis*- (roughly 23%), 9-*cis*- (25%), 13-*cis*- (4%), 7,9-di-*cis*- (11%) and many di- and tri-*cis*- conformations were formed (see inset in Fig. 1 at higher resolution). 7,9,11-Tri-*cis*-, 7,9,13-tri-*cis*-, 9,11- and 7,13-di-*cis*-production is low, 11,13-, 9,13- and 7,11-di-*cis*-isomers add up to about 3%. 9,11,13-Tri-*cis*-retinal (2.7%) is very unstable and probably decays to 9,13-di-*cis*-retinal (0.1%) within ca. 30 min, whereas 7-*cis*-retinal (1.2%) is the most unstable conformation and elutes close to all-*trans*-retinal. This is proved by cochromatography of pure all-*trans*-retinal together with the just collected 7-*cis*-isomer of previous runs, when a highly concentrated isomer mixture was fractionated immediately after illuminating an all-*trans*-retinal sample. The fast decay of the 7-*cis* form is shown in Fig. 2 for some of the chromatograms during its separation and collection.

Illumination and solvent parameters

In our hands and under our conditions all-*trans*-retinal isomerizes most (67%) within the first 40 min of illumination; during further illumination for 20 to 80 min the additional production of isomers increases little (2.7 and 1.7%). The

Table 2
Elution order of retinal isomers in *n*-hexane–*tert.*-butyl methyl ether (97:3, v/v, cf. Fig. 1)

Isomers (tentatively)	Peak elution number	Elution time (min)
Unknown	E1	5.65
Unknown (7,9,11-tri-*cis*- ?)	E2	5.94
Unknown	E3	6.43
7,9,13-tri-*cis*-Retinal	E4	7.31
Unknown	E5	7.62
7,13-di-*cis*-Retinal	E6	7.86
11,13-di-*cis*-Retinal	E7	8.68
13-*cis*-Retinal	E8	9.23
9,11,13-tri-*cis*-Retinal	E9	9.55
9,13-di-*cis*-Retinal	E10	9.94
11-*cis*-Retinal	E11	10.61
9,11-di-*cis*-Retinal	E12	11.00
7,11-di-*cis*-Retinal	E13	11.61
9-*cis*-Retinal	E14	12.10
Unknown	E15	12.62
7,9-di-*cis*-Retinal	E16	13.96
7-*cis*-Retinal	E17	15.48
Unknown	E18	16.17
all-*trans*-Retinal	E19	16.83

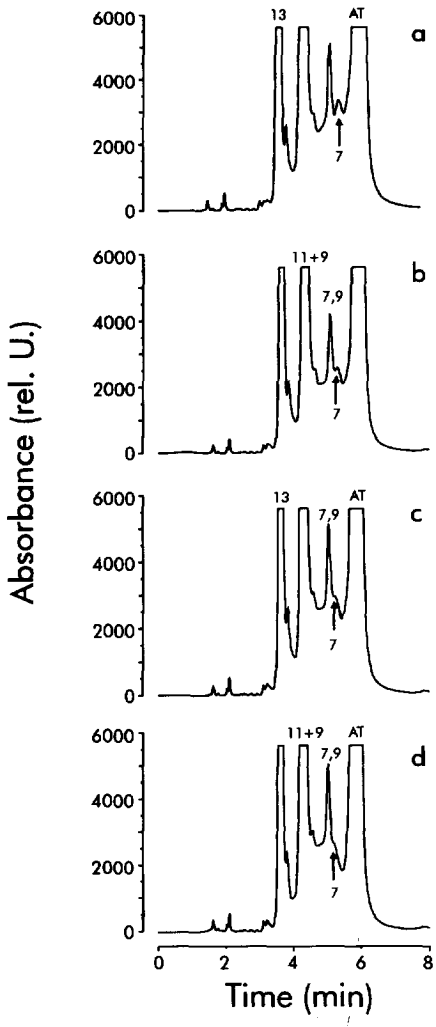

Fig. 2. Fast decay of the 7-*cis*-retinal (marked with ↑) during its separation and collection from a highly concentrated retinal isomer mixture: 28 min between measurement of (a) and (b), 14 min between (b) and (c), 48 min between (c) and (d). Elution in *n*-hexane–*t*BME (90:10, v/v), 2 ml/min, 53 bar, 371 nm, 0.02 AUFS, DuPont column. Main peaks 13-*cis*-, 11-*cis*- and 9-*cis*- (coeluting), 7,9-di-*cis*- and all-*trans*-retinal are saturated.

production of the 11-*cis*-retinal peak occurs during the first 40 min (23.3%); longer illumination (60 or 120 min) produces less 11-*cis*-retinal (23 or 21%), but leads to a small increase of 11,13-di-*cis*-, 9,11,13-tri-*cis*- and 9,13-di-*cis*-retinal.

Since the production of isomers depends on parameters such as the wavelength and time of the isomerizing light or the polarity of the solvents, we compared also solvents like ethanol and *n*-hexane with 2,2,2-trifluoroethanol.

The solvent ethanol leads after 40 min illumination to a relatively high degree of 11-*cis*- (28%) and 13-*cis*- (18%), and less 9-*cis*- (15%) and 7,9-di-*cis*-retinal (2.5%). After 40 min we get in ethanol seven and in 2,2,2-trifluoroethanol ten isomers. The percentage of 9-*cis*-retinal is doubled in 2,2,2-trifluoroethanol. In ethanol 11-*cis*-production is somewhat higher than in 2,2,2-trifluoroethanol and 13-*cis*-retinal amounts to 18%, but only to 4.7% in 2,2,2-trifluoroethanol.

In *n*-hexane isomerization by light is not efficient, only 13-*cis*- (7%) and 9-*cis*-retinal (2%) are formed, negligible are the 11-*cis*- or 7,9-di-*cis*-isomers; the rest remains as all-*trans*-retinal (86%).

Assignment of peaks

The main peaks 13-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-retinal were confirmed by cochromatography with samples of authentic material in crystalline form. In addition, the ^{1}H NMR spectra were measured; the values of the main peaks are listed in Table 3 and are in good agreement with data found in the literature [20]. In this table the chemical shifts for 9-*cis*- and 11-*cis*-retinal and the chemical shifts and coupling constants for 9-*cis*- and 11-*cis*-retinol, not found in the literature sofar, are given. As an example, Fig. 3 depicts a ^{1}H NMR spectrum of a 13-*cis*-retinal sample, used for cochromatography throughout this paper.

9,13-Di-*cis*-retinal was identified either by comparison of the matched UV absorption spectrum of the sample with reference spectra (Sperling, unpublished) or by comparison of the retention time with reported values from Sperling et al. [10].

The remaining di-*cis*- and tri-*cis*-isomers basically elute like in Bruening et al. [11]; small differences are found, which might be caused by the different mobile phase and bleaching procedure. They separated the isomers with the unusual eluent 1,1,2-trichlorotrifluoroethane (freon 113)–*tert*.-butyl methyl ether (97:3, v/v).

Table 3
Chemical shift δ_H and coupling constant J of retinal and retinol isomers in C^2HCl_3, calibration with tetramethylsilane

Proton		Retinal				Retinol			
		all-*trans*	9-*cis*	11-*cis*	13-*cis*	all-*trans*	9-*cis*	11-*cis*	13-*cis*
$1,1'CH_3$	δ_H	1.03 [s]	1.05 [s]	1.02 [s]	1.03 [s]	1.03 [s]	1.01 [s]	1.03 [s]	1.00 [s]
$5CH_3$	δ_H	1.72 [s]	1.76 [s]	1.72 [s]	1.72 [s]	1.72 [s]	1.72 [s]	1.71 [s]	1.68 [s]
$9CH_3$	δ_H	2.02 [s]	2.03 [s]	1.99 [s]	2.03 [s]	1.96 [s]	?1.93 [s]	1.93 [s]	1.94 [s]
$13CH_3$	δ_H	2.33 [s]	2.32 [s]	2.36 [s]	2.15 [s]	1.87 [s]	?1.83 [s]	1.90 [s]	1.91 [s]
2H	δ_H	1.47 [m]	1.50 [m]	1.47 [m]	1.48 [m]	1.47 [m]	1.45 [m]	1.46 [m]	1.44 [m]
3H	δ_H	1.62 [m]	1.64 [m]	1.62 [m]	1.61 [m]	1.62 [m]	1.60 [m]	1.60 [m]	1.58 [m]
4H	δ_H	2.03 [m]	2.06 [m]	2.02 [m]	2.03 [m]	2.03 [m]	2.02 [m]	2.01 [m]	1.99 [m]
7H	δ_H	6.34 [d]	6.33 [d]	6.34 [d]	6.35 [d]	6.18 [d]	6.15 [d]	6.19 [d]	6.16 [d]
	J	16.2	16.0	15.4	16.0	16.0	15.9	16.0	16.2
8H	δ_H	6.16 [d]	6.66 [d]	6.15 [d]	6.18 [d]	6.11 [d]	6.60 [d]	6.10 [d]	6.08 [d]
	J	16.2	16.0	15.4	16.0	16.0	15.9	16.0	16.2
10H	δ_H	6.19 [d]	6.09 [d]	6.54 [d]	6.22 [d]	6.11 [d]	5.98 [d]	6.53 [d]	6.10 [d]
	J	15.0	11.5	11.3	11.3	11.2	11.5	12.3	10.3
11H	δ_H	7.14 [dd]	7.22 [dd]	6.69 [dd]	7.04 [dd]	6.62 [dd]	6.68 [dd]	6.35 [dd]	6.64 [dd]
	J	15.0; 11.5	15.0; 11.5	11.3; 11.3	14.8; 11.3	15.2; 11.2	15.0; 11.5	12.3; 11.4	15.0; 10.3
12H	δ_H	6.37 [d]	6.30 [d]	5.93 [d]	7.28 [d]	6.29 [d]	6.19 [d]	5.87 [d]	6.57 [d]
	J	15.0	15.0	11.3	14.8	15.2	15.0	11.4	15.0
14H	δ_H	5.97 [d]	5.97 [d]	6.09 [d]	5.84 [d]	5.68 [t]	5.65 [t]	5.71 [t]	5.54 [t]
	J	8.2	8.3	8.2	8.0	7.1	7.2	7.0	7.2
15H	δ_H	10.11 [d]	10.10 [d]	10.08 [d]	10.20 [d]	4.32 [d]	4.27 [d]	4.29 [d]	4.29 [d]
	J	8.2	8.3	7.7	8.0	7.2	7.2	7.0	7.2

The protons are numbered according to the structure scheme (inset) in Fig. 3. Chemical shift $\delta_H \pm 0.03$, coupling constant $J \pm 0.5$ Hz. [s] = singlet, [m] = multiplet, [d] = doublet, [dd] = doublet doublet, [t] = triplet.

In Table 2, 6 of 19 retinal isomers remain unidentified, in Bruening et al. [11] 5 of 15. Peak detection was in the range of 0.1 ng all-*trans*-retinal.

Eluent ratio (v/v)

With the mobile phase *n*-hexane–*t*BME (97:3, v/v) the isomers can satisfactorily be separated in less than 18 min at a flow-rate of 2 ml/min and 60 bar pressure. All-*trans*-retinal and the *cis*-isomers can be distinguished beginning at a ratio of 80:20 (v/v), but then 11-*cis*- and 9-*cis*-retinal and 13-*cis*- and 9,13-di-*cis*-retinal are close together. At 95:5 (v/v) are 11-*cis*- and 9-*cis*-retinal separated down to baseline. At 96:4 (v/v) 7,11-di-*cis*-becomes visible between 11-*cis*- and 9-*cis*-retinal and at 97:3 (v/v) the two isomers 9,11,13-tri-*cis*- and 9,13-di-*cis*-retinal between 13-*cis*- and 11-*cis*-retinal. Lowering the fraction of *t*BME from 20 to 3% improves the resolution, but the elution time increases at the same time from 3.7 to about 18 min at 2 ml/min (pressure ca. 55 bar, see Table 4).

n-Hexane–1,4-dioxane

Fig. 4 shows a retinal isomer mixture separated with the mobile phase *n*-hexane–1,4-dioxane (99:1, v/v). The elution order is the same as in *n*-hexane–*t*BME, the run takes 16 min. Identification was performed by cochromatography either with single isomers, separated from chromatograms above, or with solutions of pure crystals, like 11-*cis*-retinal in Fig. 4b. At 6% 1,4-dioxane 11-*cis*-, 9-*cis*- and 7,9-di-*cis*-retinal coelute, the complete retinal elution is between 3 and 5 min at 2 ml/min flow-rate (77 bar).

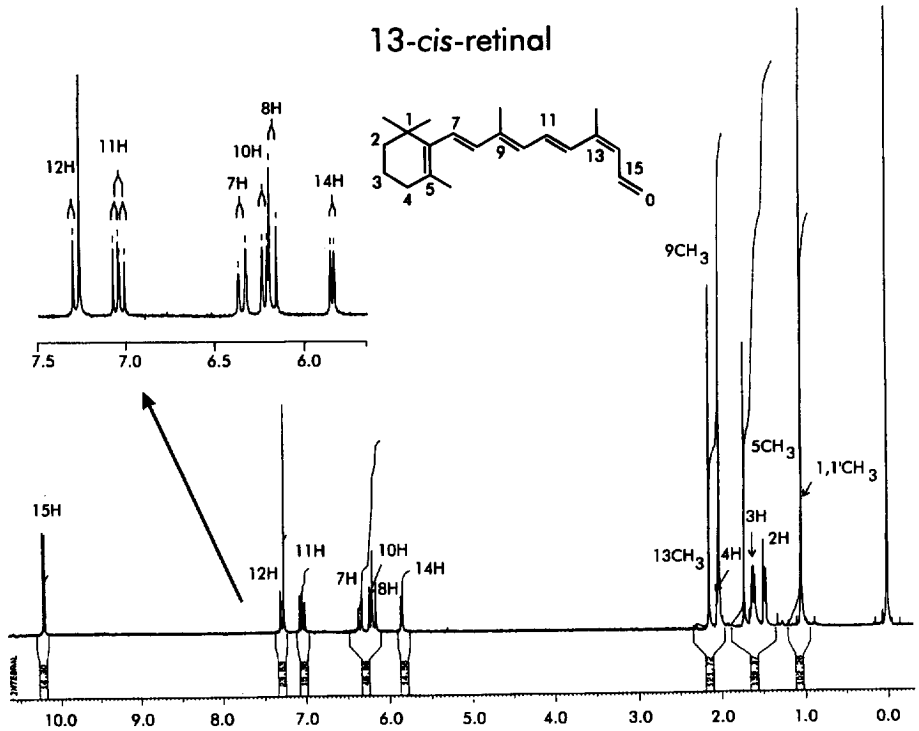

Fig. 3. 400 MHz proton magnetic resonance spectrum of 13-*cis*-retinal in C^2HCl_3, calibration with tetramethylsilane. The protons are numbered according to the structure scheme (inset). X-axis indicates δ_H.

n-Heptane–tert.-butyl methyl ether

Separation of 13-*cis*-, 11- and 9-*cis*- (together), 7,9-di-*cis*- and all-*trans*-retinal isomers with the mobile phase *n*-heptane–*tert.*-butyl methyl ether begins at a ratio of 92:8 (v/v); the run takes only 6.5 min (2 ml/min, 91 bar). In order to distinguish 11-*cis*- from 9-*cis*-retinal, the fraction of *t*BME has to be lowered below 2%, which at the

Table 4
Separation of retinal isomers at different ratio of mobile phase, flow-rate (ml/min), pressure (bar)

Mobile phase			Elution time (in min) for		Separation of 11-*cis*- and 9-*cis*-retinal peak		
Ratio	ml/min	bar	Start	End of run	*N* (11-*cis*)	α	R_s
80:20	2	56	2.2	3.7	not separated	1	0
88:12	2	58	3.0	5.2	not separated	1	0
90:10	2	52	3.4	5.8	not separated	1.04	0.48
95:5	2	54	5.8	11.0	9460	1.11	1.79
96:4	2	54	6.0	15.0	9450	1.15	2.48
97:3	2	60	6.0	20.0	10180	1.19	3.07

Mobile phase is *n*-hexane–*tert.*-butyl methyl ether, plate number $N = 16(t_{R(11\text{-}cis)}/w)^2$, selectivity ($\alpha$) and resolution ($R_s$) referring to 11-*cis*- and 9-*cis*-retinal peak (DuPont column Zorbax SIL).

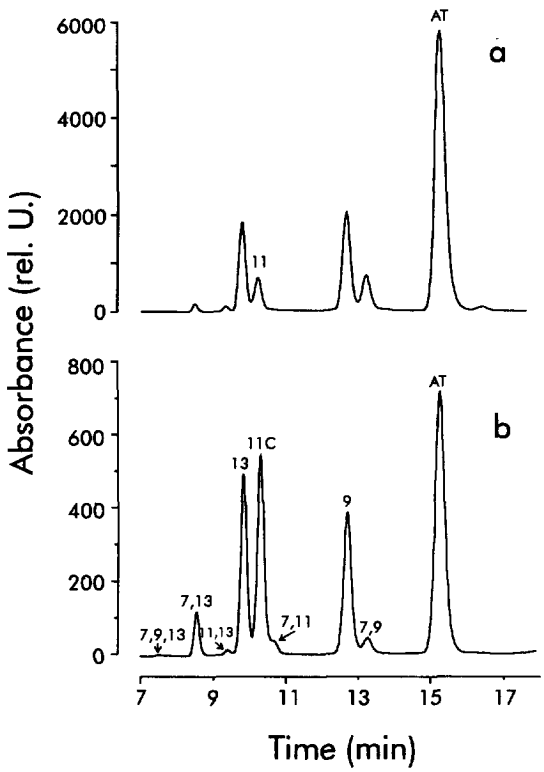

Fig. 4. Chromatogram of retinal isomers with the mobile phase *n*-hexane–1,4-dioxane (99:1, v/v), flow-rate 2 ml/min, 69 bar, measuring wavelength 371 nm, 0.02 AU. (a) Elution order (retention time in min): 7,9,13-tri-*cis*- (7.44), 7,13-di-*cis*- (8.30), 11,13-di-*cis*- (9.25), 13-*cis*- (9.77), 11-*cis*- (10.20), 7,11-di-*cis*- (10.80), 9-*cis*- (12.70), 7,9-di-*cis*- (13.47) and all-*trans*-retinal (15.54). (b) Cochromatography of 11-*cis*-retinal (11C) with a retinal sample like in (a). DuPont column.

same time leads to an elution time of more than 16 min and brings implications for the HPLC mixing device of many systems. A complete and satisfying chromatogram of retinal isomers with this mobile phase will be described in an forthcoming paper.

3.2. Retinol isomers

n-Hexane–*tert.*-butyl methyl ether

If the mixture of retinal isomers, formed in ethanol or in 2,2,2-trifluoroethanol, was reduced to a retinol isomer mixture, up to 15 retinol isomers could be separated; 12 are tentatively assigned in Fig. 5 and in Table 5. The mobile phase for this chromatogram was *n*-hexane–*t*BME (93:7, v/v; 4 ml/min; 119 bar; 325 nm). (7,9,13-tri-*cis*-Retinol, 11,13-di-*cis*-, 9,11-di-*cis*-, 7,11-di-*cis*- and 7,9-di-*cis*-retinol from Table 5 are not resolved in this cochromatogram at this magnification, but are deduced from separate experiments, described elsewhere [15].) Reduction of retinal by $NaBH_4$ to retinol was more than 85%.

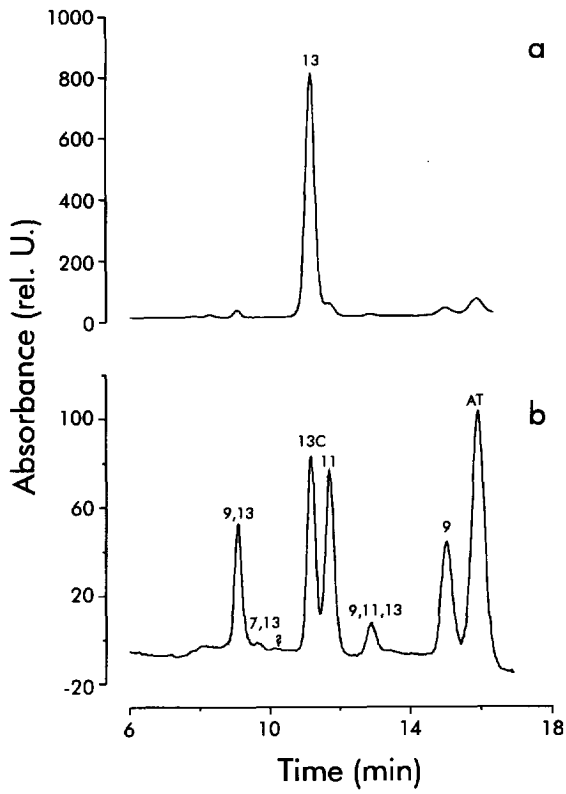

Fig. 5. Chromatogram of retinol isomers. Mobile phase: *n*-hexane–*t*BME (93:7, v/v); flow-rate 4 ml/min; 119 bar; 325 nm; elution order see Table 5. (a) 13-*cis*-Retinol, (b) cochromatography (13C) of 13-*cis*-retinol with retinol isomers. 7,9,13-tri-*cis*-Retinol, 11,13-di-*cis*-, 9,11-di-*cis*-, 7,11-di-*cis*- and 7,9-di-*cis*-retinol from Table 5 are not resolved in this cochromatogram at this magnification but are deduced from separate experiments, described elsewhere [15]. Reduction of retinal by $NaBH_4$ to retinol was more than 85%. DuPont column.

Table 5
Elution order of retinol isomers in *n*-hexane–*tert.*-butyl methyl ether (93:7, v/v, cf. Fig. 5)

Peak elution number	Isomers	Elution time (min)	Elution number of corresponding retinal isomer
E1	7,9,13-tri-*cis*-retinol	8.02	E4
E2	9,13-di-*cis*-retinol	9.08	E10
E3	7,13-di-*cis*-retinol	9.60	E6
E4	unknown isomer	10.17	E5
E5	13-*cis*-retinol	11.15	E8
E6	11,13-di-*cis*-retinol	11.48	E7
E7	11-*cis*-retinol	11.69	E11
E8	9,11-di-*cis*-retinol	12.87	E12
E9	9,11,13-tri-*cis*-retinol	13.38	E9
E10	7,11-di-*cis*-retinol	14.42	E13
E11	9-*cis*-retinol	15.02	E14
E12	7,9-di-*cis*-retinol	15.56	E16
E13	all-*trans*-retinol	15.91	E19

Assignment of peaks

As can be seen from Table 5, the elution pattern changes for the various isomers (compare elution number of retinal peak). It was verified by reduction of the single retinal isomers in Table 2 and cochromatography of its retinol form. The main isomers all-*trans*-, 9-*cis*-, 11-*cis*- and 13-*cis*-retinol were again identified by chromatography of authentic material, by UV absorption and ^{1}H NMR measurement, e.g. 13-*cis*-retinol in Fig. 5 and Table 3. Identification of the di-*cis*- and tri-*cis*-conformations was quite complicated, since we experienced that some of the short-living and very unstable isomers decayed into other isomers before or because of the reduction step (for instance 9,11,13-tri-*cis*- in 9,13-di-*cis*-retinol). Because of the varying bleaching conditions and different solvents used to produce different mixtures of retinal isomers (see above) we could distinguish by number and amount which retinol isomers were formed after reduction and their individual fate thereafter.

The following seven peaks were assigned tentatively: 7,13-di-*cis*-, 11,13-di-*cis*-, 9,11-di-*cis*- (stable), 7,11-di-*cis*-, 7,9-di-*cis*-, 7,9,13-tri-*cis*-, 9,11,13-tri-*cis*-retinol (unstable). There are no known chromatograms for these retinol isomers published before. Four peaks at the beginning of the elution presumably also belong to the retinol family, but they remained unidentified.

Eluent ratio (v/v)

Retinol isomers could be separated in *n*-hexane–*t*BME if the ratio of the solvents ranged from 70:30 up to 96:4 (v/v). At 30% *t*BME a run takes around 9 min at 2 ml/min flow-rate (56 bar); 9,13-di-*cis*- elutes at 5 min, 13-*cis*- and 11-*cis*-retinal coelute at 6–6.2 min, and 9-*cis*- and all-*trans*-retinal are half separated (8 and 8.5 min). By lowering the amount of *t*BME to 20 or 15%, 13-*cis*- and 11-*cis*- begin to separate half or to two-thirds, the elution starts at 8 (respectively 11) min with 9,13-di-*cis* and ends after 15 (respectively 21) min with all-*trans*-retinal. Increasing the flow-rate to 3 ml/min (87 bar), the retinals elute between 7 and 12.5 min. At 10% *t*BME mainly 13-*cis*- and 11-*cis*- are influenced and almost separated, but then the run takes 30 min at 2 ml/min flow-rate (52 bar). By increasing the flow-rate to 3 (82 bar), 3.5 (98 bar) and 4 ml/min (114 bar) the run shortens to 19, 16 and 13 min, which fulfils most chromatographic tasks. For optimal separation of all the di-*cis*- and tri-*cis*-isomers the amount of *t*BME has to be lowered further to 7% (see Fig. 5 and Table 5) or even 6%. A lower proportion of *t*BME does not

separate 13-*cis*- and 11-*cis*-retinal anymore and the complete run lasts longer than 30 min, which is too long for practical reasons.

n-Hexane–1,4-dioxane

We also tested the solvent *n*-hexane–1,4-dioxane; a typical chromatogram is shown in Fig. 6 (95:5, v/v). It is of great importance to notice that this eluent reverses the elution order to 9,13-di-*cis*-, 7,13-di-*cis*-, 11-*cis*-, 13-*cis*-, 9,11,13-tri-*cis*-, 9-*cis*- and all-*trans*-retinol. This is true for ratios of 88:12 to 99:1 (v/v). At 12% 1,4-dioxane the retinol isomers elute from 4.5 to 6.7 min at 2

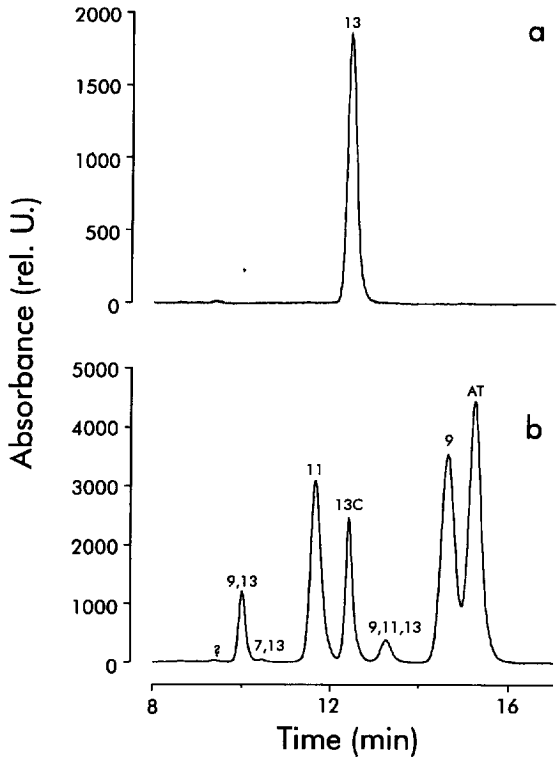

Fig. 6. Chromatogram of retinol isomers with the mobile phase *n*-hexane–1,4-dioxane (95:5, v/v), flow-rate 2 ml/min, 50 bar, 325 nm, elution order (retention time in min): 9,13 = 9,13-di-*cis*-retinol (10.00), 7,13 = 7,13-di-*cis*-retinol (10.43), 11 = 11-*cis*-retinol (11.66), 11,13 = 11,13-di-*cis*-retinol (12.00, hidden by 11-*cis*-), 13 = 13-*cis*-retinol (12.41), 9,11,13 = 9,11,13-tri-*cis*-retinol (13.27), 9 = 9-*cis*-retinol (14.64), AT = all-*trans*-retinol (15,25). (a) Pure 13-*cis*-retinol, (b) cochromatography (13C) of 13-*cis*-retinol with other retinol isomers. DuPont column. Note: elution of 11-*cis*-retinol and 13-*cis*-retinol is inverted compared to Fig. 5!

ml/min flow-rate, 9-*cis*- and all-*trans*- only being separated one-third. They are separated about half from 12 to 8%, good from 7 to 6% and best from 5 to 2%, but at the same proportions the elution time increases from 6.5 (respectively 9.5) to 12 and to 18 (respectively 38) min. The optimum for separation of 11-*cis*- and 13-*cis*- lies between 12 and 3% 1,4-dioxane, at 2% they are close (27.8 and 29.0 min, respectively) and at 1% they coelute. For a Merck column the elution pattern is similar, with only small changes for 11-*cis*- and 13-*cis*-retinal, which get closer. The mobile phase *n*-hexane–1,4-dioxane was commonly used, but since 1,4-dioxane is supposed to be toxic or even carcinogenic, the elution order of the smaller di-*cis*- and tri-*cis*-isomers was not studied further.

Identification of the main peaks was performed again with authentic material like above; cochromatograms of the isomer mixture with 13-*cis*-retinol are shown in Figs. 6a and 6b.

n-Heptane–tert.-butyl methyl ether

Fig. 7 represents a chromatogram with the eluent system *n*-heptane–*t*BME (92:8, v/v; flow-rate 3 ml/min; 139 bar). These solvents are less toxic and give the best separation among the solvents we tested. The complete run is reasonably short. The elution order is the same as in *n*-hexane–*t*BME, but 11-*cis*- and 13-*cis*-retinol are inverted compared to the eluent system *n*-hexane–1,4-dioxane. Increasing the proportion of *t*BME from 6 to 7 or 8%, the elution time increases from 13.06 to 17.85 to 21.5 min (3 ml/min; 136–140 bar) for the maximum of all-*trans*-retinol. At 7% *t*BME and 4 ml/min flow-rate (187 bar) the run lasts 11.25 min for all-*trans*-retinol. Fig. 7b shows a good compromise.

3.3. Columns

During this study two columns, DuPont Zorbax SIL and Merck LiChroCART (250-4), with small-diameter (5–6 μm) porous spherical silica particles were used. There are only small differences, but the Merck LiChroCART column showed better separation for retinal isomers, especially the 9-*cis*- and 11-*cis*-isomers, com-

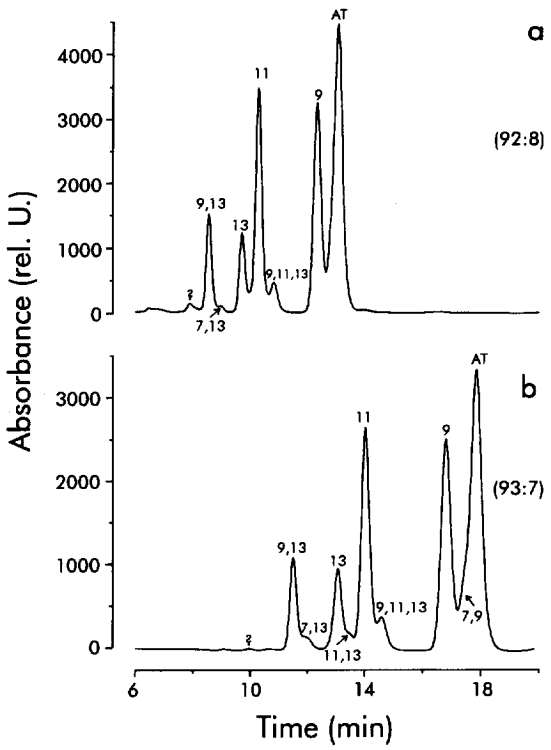

Fig. 7. Chromatogram of retinol isomers with the mobile phase *n*-heptane–*t*BME in (a) (92:8, v/v), flow-rate 3 ml/min, 139 bar, 325 nm and in (b) (93:7, v/v), 3 ml/min, 136 bar. Elution order [retention time in min for (a); (b)]: 9,13 = 9,13-di-*cis*-retinol (8.55; 11.50), 7,13 = 7,13-di-*cis*-retinol (8.96; 11.90), 13 = 13-*cis*-retinol (9.70; 13.06), 11,13-di-*cis*-retinol (–; 13.41), 11 = 11-*cis*-retinol (10.26; 14.00), 9,11,13 = 9,11,13-tri-*cis*-retinol (10.78; 14.60), 9 = 9-*cis*-retinol (12.31; 16.80), 7,9-di-*cis*-retinol (12.75; 17.33, hidden by AT) and AT = all-*trans*-retinol (13.07; 17.82). DuPont column. Note: elution of 11-*cis*-retinol and 13-*cis*-retinol is inverted compared to Fig. 6, but the same as in Fig. 5!

pared to the DuPont Zorbax SIL, when exactly the same eluent was used. The DuPont column, on the other hand, was more suitable for separating retinol isomers. Even better separation is achieved using two DuPont columns in series [10].

4. Discussion

The most extensive studies on retinal isomers before ours originate from Bruening et al. [11], who described 14 retinal isomers and 5 more polar unidentified isomers. They obtained retinal isomers by irradiation of all-*trans*-retinal in acetonitrile, chromatography was performed with the uncommon mobile phase freon 113 (1,1,2-trichlorotrifluoroethane)–*tert.*-butyl methyl ether (97:3, v/v). We used for the first time 2,2,2-trifluoroethanol, which seems to be superior for creating different retinal isomers by irradiation of all-*trans*-retinal for 120 min, and we could resolve up to 19 isomers with the eluent *n*-hexane–*t*BME on the DuPont column.

2,2,2-Trifluoroethanol has a strong dipole. Because of its acid, free movable protons, it is more polar than ethanol and we assume that, consequently, the isomerization of 11-*cis*- and especially that of 13-*cis*-retinal in di-*cis*- and tri-*cis*-isomers is much more favoured in 2,2,2-trifluoroethanol than in ethanol. According to Denny and Liu [21] the appearance of steric hindered isomers is favoured in solvents with a higher dielectric constant.

If it is desired to produce and resolve many conformations, the right choice of solvent for illumination is important. In *n*-hexane for instance production is very low as seen above. Production of 11-*cis*-retinal by photoisomerization might be more effective in other solvents than 2,2,2-trifluoroethanol, e.g. in acetonitrile [22]. We used 2,2,2-trifluoroethanol because we found that in a relatively short illumination time much more isomers were found than in other known solvents. Additionally, 11-*cis*-retinal is relatively stable in this solvent. Our intention was also to establish a simple method for producing many isomers and to identify unknown samples in an easy way from our own standard chromatograms.

Formation of 7-*cis*-retinal depends very much on the solvent. Denny and Liu [21] obtained more 7-*cis*-retinal by illumination in acetonitrile than in ethanol, but from the literature we know that 7-*cis*-retinal decays rapidly into other di-*cis*- and tri-*cis*-conformations [23,24]. Hence the stability of the different isomers in various solvents has to be considered too [8]. 9,11,13-Tri-*cis*-retinal is very unstable [25], 9,13-di-*cis*- changes to 9-*cis*- and 13-*cis*-retinal upon longer illumination [26] and 9,11,13-tri-*cis*-retinal con-

verts to 9,13-di-*cis*-retinol according to Knudsen et al. [27]. For ^{1}H NMR identification at reasonable measuring times it would be necessary to start with high amounts of these isomers on the retinal side to collect fractions of about 1 mg on preparative columns, but this is tedious to accomplish.

The retinal-isomers can satisfactorily be separated in less than 18 min (Fig. 1) with *n*-hexane–*t*BME. Changing the eluent ratio as described above influences the resolution and the consumption of solvents and the ratio depends on the actual problem to be solved. The detection limit for all-*trans*-retinal is about 0.1 ng.

Chromatograms for retinol presented here show for the first time a rather complete and well-resolved separation of *trans*-, *cis*- and several di-*cis*-and tri-*cis*-isomers not previously described in the literature. It is important to emphasize that the elution order in *n*-hexane–*t*BME and also in *n*-heptane–*t*BME is 13-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-retinol, whereas in *n*-hexane–1,4-dioxane it is inverted: 11-*cis*- elutes before 13-*cis*-retinol. So only Bridges and Alvarez [8] and Fong et al. [12] assigned the chromatograms correctly. But earlier, e.g. [1], and even later, e.g. [13], publications of this group gave wrong chromatograms concerning these main isomers, which means that they were not aware of this problem. In Figs. 5, 6 and 7 identification of the peaks was performed by cochromatography and ^{1}H NMR control, as far as possible. The elution order of the di-*cis* and tri-*cis* peaks of the alcohol form of retinal was determined with the help of the assignment of the retinal isomers established above: the identified di-*cis*- and tri-*cis*-retinal isomers were separated, collected and reduced to retinols. Then they were cochromatographed both with samples containing all retinal isomers (e.g. like in Fig. 1) in the reduced form and with pure retinol samples originating from the retinal crystals. For additional discrimination different retinal mixtures, which varied by irradiation and solvent parameters and hence gave different amounts of isomers, were cochromatographed after reduction. Also the comparison of the behaviour of the two columns we used was very helpful for identification. They showed differences in small details and allowed therefore discrimination of minor or shoulder peaks. The consistency and possible appearance of unstable retinal peaks in identical or different isomeric forms after reduction to alcohol was compared and cross-checked in 600 original retinal and retinol chromatograms.

The elution time and pressure given in this paper are dependent on the columns used and our experimental setup. Most of the other authors dealing with HPLC of retinoids employed a Merck column packed with silica particles (for an overview see Ref. [15], pp. 155–156). Therefore this column was applied for comparison too. Both columns showed a different behaviour, in short: the higher peak resolution and better baseline separation for the retinol isomers was achieved on the DuPont column, whereas the Merck column was superior for separation of the retinal isomers. The reason for this finding might origin in the difference of the specific inner surface area, which is greater for the Merck LiChroCART column (650 m^2/g) compared to Zorbax SIL (300 m^2/g). However, retinal could be separated even better on DuPont columns if two of these columns were used in tandem [10], but then the complete run took more than 28 min, which often is too long for practical reasons.

With *n*-hexane–*t*BME a good baseline separation takes more than 30 min. This might not be convenient, especially if one has to measure a high number of samples. It is indicated only when all the isomeric forms have to be resolved, but not if only crude estimations are wanted, e.g. total content of vitamin A in blood samples or in food like cheese. Also, the consumption of solvents is high, 84 ml eluent at a ratio of 93:7 (v/v) or 62 ml at a ratio of 90:10 (v/v).

Although the eluent *n*-hexane–1,4-dioxane gives good separation of retinal (Fig. 4) and retinol (Fig. 6), its use should be avoided, because *n*-hexane causes a degenerating nerve disease [17] and 1,4-dioxane is supposed to be carcinogenic [16].

Employing *n*-heptane–*t*BME, we needed 66 ml at 94:6 (v/v) and 46 ml at 93:7 (v/v). Both solvents can be used without any known risk to

health [28], they show a good baseline separation and allow to resolve the main and many di-*cis*- and tri-*cis*-isomers in relatively short analysis time. Separation is even better than in *n*-hexane–*t*BME; for instance 11,13-di-*cis*- could get resolved between 11-*cis*- and 13-*cis*-retinol with the *n*-heptane–*t*BME eluent, whereas *n*-hexane–*t*BME requires parameters which lead to very long elution times.

These advantages and the fact that *n*-heptane–*t*BME showed no influence on the isomeric forms of the retinoids lead to the conclusion that this new mobile phase might be of interest for those dealing with the measurement of retinol in vision and clinical applications. This eluent could serve for own standard chromatograms of retinal and retinol, which one should create for reliable identification of unknown samples. In order to get the different isomeric forms, we recommend irradiation of all-*trans*-retinal in 2,2,2-trifluoroethanol, followed by reduction of these isomers to establish standard chromatograms for retinol and retinylpalmitate, since illumination of the all-*trans*-form of these retinoids is not effective.

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