Journal of Chromatography, 301 (1984) 65–75
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,899

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF RETINALS, RETINOLS (VITAMIN A_1) AND THEIR DEHYDRO HOMOLOGUES (VITAMIN A_2): IMPROVEMENTS IN RESOLUTION AND SPECTROSCOPIC CHARACTERIZATION OF THE STEREOISOMERS

FABIO ZONTA* and BRUNO STANCHER
Istituto di Merceologia, Università di Trieste, Via A. Valerio 6, 34127 Trieste (Italy)
(Received May 12th, 1984)

SUMMARY

A study of mobile phases for the improved high-performance liquid chromatographic resolution of retinal, 3-dehydroretinal, retinol and 3-dehydroretinol stereoisomers is described. By using 1-octanol as a phase modifier in *n*-hexane, the simultaneous separation of a complex mixture of vitamin A_1 and A_2 isomers was satisfactorily achieved, while the separation of the corresponding aldehydes required a ternary mixture of 2-propanol, dioxane and *n*-hexane. All peaks were spectroscopically characterized by recording their UV spectra, which are reported together with comparative tables of the absorbance maxima of the stereoisomers.

INTRODUCTION

The application of high-performance liquid chromatography (HPLC) to the simultaneous fractionation of some retinol and 3-dehydroretinol isomers was reported in a recent paper[1]. This separation was developed with the aim of evaluating better the vitamin A activity of food samples containing both retinol (vitamin A_1) and 3-dehydroretinol (vitamin A_2). However, little attention has been paid to the HPLC analysis of dehydroretinols, compared with extensive studies of the separation of retinols[2–5], retinals[3,6–8] and 3-dehydroretinals[7,9].

Nonetheless, there is still a need for improving both resolution and analytical knowledge in the general field of vitamin A compounds: some peaks and/or compounds were previously unresolved or ill-defined and spectrophotometric absorbance data for some isomers are still lacking or conflicting.

In this paper we describe a new mobile phase for normal-phase HPLC which allows the separation of 7-*cis*-retinol, which has never previously been achieved. Further, all mono-*cis* isomers (7-, 9-, 11- and 13-*cis*) and some di-*cis* isomers of both retinols and 3-dehydroretinols have been simultaneously separated from the two main all-*trans* compounds.

In the absence of pure standards of the 3-dehydro series, all-*trans* 3-dehydroretinal was synthesized and purified by semi-preparative HPLC.

0021-9673/84/$03.00

As our retinols and 3-dehydroretinols were derived from the reduction of the corresponding photoisomerized aldehydes, the HPLC of the aldehydes was also considered and, to a certain extent, improved with respect to previous work[3,6–9].

The absorbance spectra of all major chromatographic peaks are also reported, together with tables of absorbance maxima, including previously published data.

EXPERIMENTAL

Apparatus

The chromatographic apparatus consisted of a pump module (Series 3 liquid chromatograph; Perkin-Elmer, Norwalk, CT. U.S.A.), a variable-wavelength spectrophotometric detector (Perkin-Elmer LC 55 B), a digital scanner (Perkin-Elmer LC 55 S), a recorder (Perkin-Elmer Model 56) and, as integrator, a chromatography data station (Perkin-Elmer Sigma 15).

A guard column (5 × 0.4 cm I.D.) (Supelco, Bellefonte, PA, U.S.A.), dry-packed with 40-μm silica pellicular packing, was connected to the analytical column (25 × 0.4 cm I.D.), containing 5-μm silica Si-60 (E. Merck, Darmstadt, F.R.G.).

For semi-preparative HPLC, a Partisil-10 column (25 × 0.94 cm I.D.) (Whatman, Clifton, NJ, U.S.A.) was used.

Reagents

A pure standard of vitamin A aldehyde (all-*trans*-retinal) was purchased from Fluka (Buchs, Switzerland).

The solvents used were dioxane and 2-propanol (HPLC grade) and chloroform (freshly distilled from P_2O_5) (E. Merck), *n*-hexane (HPLC grade) (Carlo Erba, Milan, Italy) and 1-octanol, *n*-pentane, diethyl ether and absolute ethanol (Riedel de Haën, Hannover, F.R.G.).

N-Bromosuccinimide (purum, 97%; Fluka), 4-phenylmorpholine (98%; Janssen, Beerse, Belgium), hydrochloric acid (RP, 37%) and sodium borohydride (RP, Carlo Erba) and aluminium oxide, standardized according to Brockmann II–III (E. Merck), were also employed.

Synthesis of 3-dehydroretinal and isolation of the pure all-trans isomer by semi-preparative HPLC

All-*trans*-retinal was converted into 3-dehydroretinal according to Henbest *et al.*[10]. The mixture of 3-dehydroretinal isomers obtained was first purified by liquid–solid chromatography on a 25-cm alumina column which was eluted with diethyl ether–*n*-pentane (4:96). The fraction containing the main deep-orange band of the 3-dehydroretinals was then concentrated by means of a nitrogen stream, and several aliquots of 200 μl were injected into the semi-preparative column. Repeated chromatography was performed, using 2-propanol–dioxane–*n*-hexane (0.1:1:98.9) as the mobile phase at a flow-rate of 4 ml/min. The fractions corresponding to all-*trans*-3-dehydroretinal were collected, pooled and concentrated by means of a nitrogen stream. The purity of all-*trans*-3-dehydroretinal was checked and confirmed by analytical HPLC. After the total evaporation of the solvent, the crystalline 3-dehydroretinal (characterized by UV and IR spectra) was stored under nitrogen at 4°C and remained unchanged for more than 1 month.

Photolysis

Ethanolic solutions (about 100 μg/ml) of pure all-*trans*-retinal and 3-dehydroretinal were prepared separately in two spectrophotometric silica cuvettes, deaerated by bubbling nitrogen and stopcocked. The cuvettes, standing on a water-cooled iron plate, were irradiated from a distance of 10 cm using a fluorescent bulb (18 W, 750 lumen; Philips, Eindhoven, The Netherlands). The time of photolysis was set at 4 h on the basis of previous experience[1]; the photolysis was carried out in ethanol because in apolar solvents (*e.g.*, *n*-hexane) the photolytic process does not yield all isomers (*e.g.*, 7-*cis*, 11-*cis*).

Aliquots of the ethanolic solutions were evaporated to dryness with a nitrogen stream and the residue, dissolved in *n*-hexane, was used to obtain the chromatogram of retinal and 3-dehydroretinal isomers.

Reduction process

The photolysed ethanolic solutions of retinal and 3-dehydroretinal isomers (about 100 μg/ml) were treated with sodium borohydride, performing the reduction of the aldehyde group according to Hubbard *et al.*[11], except that the amount of sodium borohydride used was reduced more than 10-fold to about 1 mg per 100 μg of aldehyde.

After the reduction, the samples were evaporated to dryness by means of a nitrogen stream; the residues were dissolved in *n*-hexane and the solutions were filtered using a syringe equipped with a small Millipore filter holder. These solutions were used to obtain the chromatograms of retinols and 3-dehydroretinols.

RESULTS AND DISCUSSION

Aldehydes

Samples of retinal and 3-dehydroretinal isomer mixtures in *n*-hexane solution were chromatographed using several mobile phases. 1-Octanol, 2-propanol and dioxane, and their mixtures, were considered as possible phase modifiers to be mixed with *n*-hexane. Some of the more interesting results are briefly as follows.

1-Octanol (1%), which possesses a higher selectivity towards retinols (as will be shown later), displays the interesting effect of differentiating well the retention times of 9-*cis*-, 7-*cis*- and all-*trans*-aldehydes, but fails to resolve the 11-*cis*/13-*cis* pair. 2-Propanol (0.2%) separates well all mono-*cis* isomers, but does not resolve the di-*cis* isomers. Dioxane (2%) gives a satisfactory resolution of the di-*cis* isomers, especially for the dehydroretinal pair 9, 13-di-*cis*/13-*cis*, the separation of which is critical and difficult to achieve with other solvent systems. However, it fails to resolve the 7-*cis*/9-*cis* isomer pair.

The best resolution resulted with 2-propanol–dioxane–*n*-hexane (0.1:1:98.9), and the chromatograms obtained by means of this mobile phase are shown in Fig. 1 for (a) retinals and (b) 3-dehydroretinals; both series display similar chromatographic behaviour. The isomer resolutions obtained by using this mobile phase are better than those previously published in which diethyl ether was used as a modifier[6–9].

The elution order of retinal isomers appears to be in close agreement with that reported by Liu and Asato[8]; one more peak was detected, eluting earlier than the

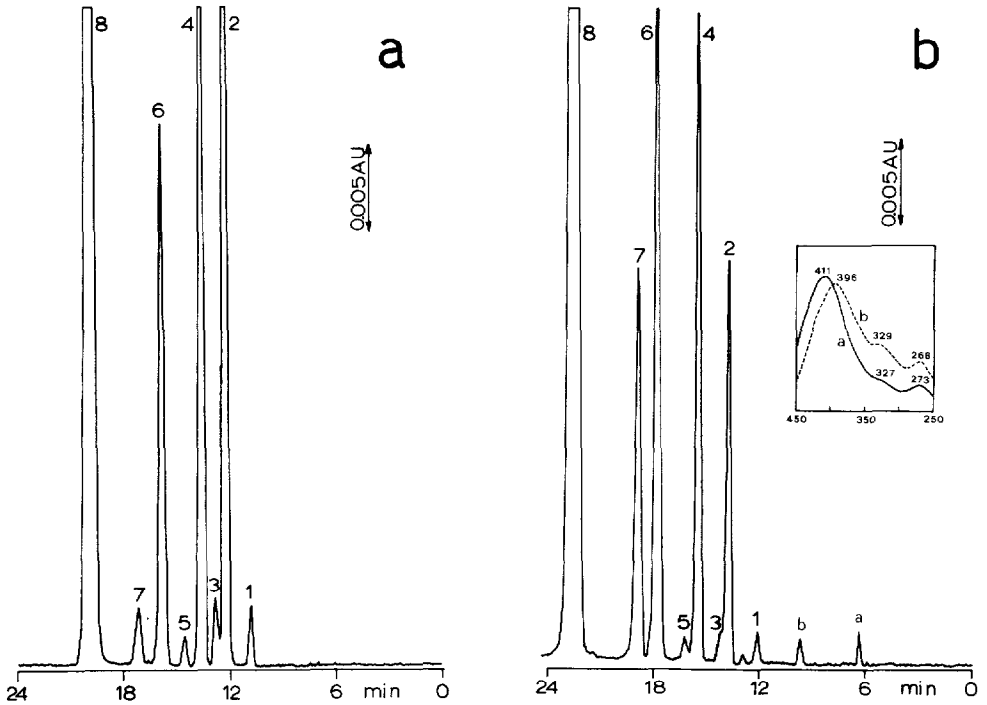

Fig. 1. Chromatograms of (a) retinal and (b) 3-dehydroretinal isomers obtained from photolysis of all-*trans*-retinal and all-*trans*-dehydroretinal. Peaks: 1 = 11,13-di-*cis*; 2 = 13-*cis*; 3 = 9,13-di-*cis*; 4 = 11-*cis*; 5 = 9,11-di-*cis*; 6 = 9-*cis*; 7 = 7-*cis*; 8 = all-*trans*. Mobile phase, *n*-hexane–dioxane–isopropanol (98.9:1:0.1); flow-rate, 1 ml/min; detection wavelength, 370 nm. Absorption spectra corresponding to unknown dehydroretinals (a and b) are also reported.

13-*cis*-retinal, and its spectrum, with a peculiar shape and an absorbance maximum at 302 nm, is shown in Fig. 2 (where the spectra of all resolved retinals, obtained by means of the stop-flow method, are also reported. Recently, Chandraratna *et al.*[12] reported a value of 302 nm as the wavelength of maximum absorbance of 11,13-di-*cis*-retinal (and also of 9,11,13-tri-*cis*-retinal), and explained the anomalous shift to the blue as being due to the highly hindered configuration of such isomers, which are therefore assumed to exist mainly in a 12-*s*-*cis* configuration. Evidence for our attribution of the 11,13-di-*cis* configuration to the first-eluting aldehyde was obtained by collecting this compound alone, reducing it to the corresponding alcohol and checking its new retention time and absorbance spectrum, which actually coincided with those possessed by 11,13-di-*cis*-retinol.

The other retinals were identified by comparison of our recorded absorbance data with those available in the literature[6,8,11–13]. All such data are summarized in Table I. For the 9,11-di-*cis*- and 9,13-di-*cis*-retinal isomers, the close resemblance of their UV spectra does not allow positive peak identification. Therefore, we relied on the elution order and the peak attribution reported by Liu and Asato[8]. In order to conform to such an attribution, the 9,11-di-*cis* configuration must be assigned to the peak identified as 9,13-di-*cis* in a previous paper[1].

Peak identities of 3-dehydroretinal isomers were obtained on the same basis

TABLE I

ABSORPTION MAXIMA OF RETINAL AND 3-DEHYDRORETINAL ISOMERS IN *n*-HEXANE–DIOXANE–ISOPROPANOL (98.9:1:0.1) COMPARED WITH LITERATURE DATA[6–8,11–13]

Isomer	*Retinals*			*3-Dehydroretinals*		
	This work	*n-Hexane*	*Ethanol*	*This work*	*n-Hexane*	*Ethanol*
All-*trans*	373	368[8]	383[8] 381[13]	389, 308sh	385[11] 387, 305sh[7]	401, 314sh[13] 400, 308sh[7]
7-*cis*	362	359[8]	377[8]	364	–	378[7]
9-*cis*	363, 290, 250	363[8] 359[6]*	373[13]	380, 311	–	391, 315[13] 390, 313[7]
11-*cis*	365, 289, 253	365[8] 362, 250[6]*	379.5[11] 376.5, 290, 254[13]	385, 317sh, 253	–	393, 321sh, 252[13] 393, 314, 250[7]
13-*cis*	367, 256	363[8] 361[6]*	375, 257[13]	386, 309sh	–	395, 314sh[13] 394, 307sh[7]
9,11-Di-*cis*	356, 291, 231**	352[8]	368[8]	379, 309sh, 253**	–	–
9,13-Di-*cis*	359, 290, 231**	359[8]	368[13]	375, 330sh, 254sh**	–	–
11,13-Di-*cis*	302, 226	356[8]	373[13] 302[12]	358, 270, 262	–	386, 269, 261[13]

* Diethyl ether–*n*-hexane (12:88) as solvent.
** Dioxane–*n*-hexane (2:98) as solvent.

TABLE II

ABSORPTION MAXIMA OF RETINOL AND 3-DEHYDRORETINOL ISOMERS IN *n*-HEXANE–1-OCTANOL (96:4) COMPARED WITH LITERATURE DATA[7,11,13]

Isomer	*Retinols*			*3-Dehydroretinols*		
	This work	*Literature data*		*This work*	*Literature data*	
		n-Hexane	*Ethanol*		*n-Hexane*	*Ethanol*
All-*trans*	326	325[11]	325[13]	352, 288, 278sh, 234	–	350, 286, 276[13] 352, 287, 276[7]
7-*cis*	322	–	–	331, 289, 279sh	–	329, 288, 278[7]
9-*cis*	323, 258	–	323, 258[13]	350, 288, 278sh, 239	–	348, 287, 277[13] 347, 286, 277[7]
11-*cis*	322, 236	318[11]	319, 233[13]	347, 287, 279sh, 232	–	344, 286, 278sh[13] 346, 286, 277[7]
13-*cis*	328	–	328[13]	355, 289, 278sh, 232	–	352, 288, 277[13] 352, 287, 276[7]
9,11-Di-*cis*	326, 258sh	–	–	352, 289, 278sh, 242	–	–
9,13-Di-*cis*	–	–	324, 263[13]	–	–	350, 288, 280sh[13]
11,13-Di-*cis*	312, 238	–	312[11] 311[13]	346, 290sh, 277, 232	–	337, 290sh, 277[13]

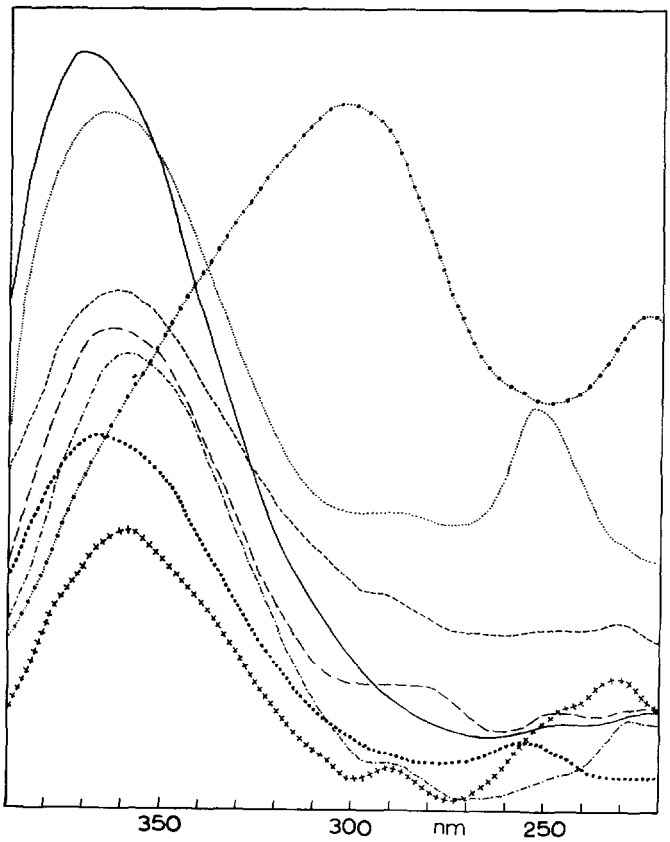

Fig. 2. Absorption spectra of retinal isomers: ———, all-*trans*; ---------, 7-*cis*; – – –, 9-*cis*; ....., 11-*cis*; ....., 13-*cis*; .–.–.–., 9,13-di-*cis*; ●...●...●, 11,13-di-*cis*; + + +, 9,11-di-*cis*. Spectra were recorded in an 8-μl spectrophotometer cell by means of the stop-flow method. Absorbance scale in arbitrary units. Solvent: *n*-hexane–dioxane–isopropanol (98.9:1:0.1).

as above, and their UV spectra are reported in Fig. 3. The spectrum of peak 3 (which in Fig. 1b appears as a shoulder on the 13-*cis*-3-dehydroretinal peak) was obtained from another chromatographic run employing dioxane–*n*-hexane (2:98) as the mobile phase, in which case the peak is completely resolved from that of 13-*cis*-3-dehydroretinal. We have no data for identifying the two peaks a and b eluting earlier than 11,13-di-*cis*-3-dehydroretinal, whose absorbance maxima do not correspond to any of those previously reported, but which have spectral shapes similar to those of the 3-dehydroretinal isomers (see Fig. 1b).

Alcohols

Samples of the photoisomerized aldehydes (retinals and 3-dehydroretinals) were reduced to the corresponding alcohols and chromatographed employing as the mobile phases (a) 1-octanol–*n*-hexane (4:96) and (b) 2-propanol–*n*-hexane (0.4:99.6). The retinol chromatograms obtained are reported in Fig. 4a and b, respectively. The higher selectivity of 1-octanol appears evident, and its use as an effective phase modifier allowed us to resolve for the first time 7-*cis*-retinol from 9-*cis*- and all-*trans*-

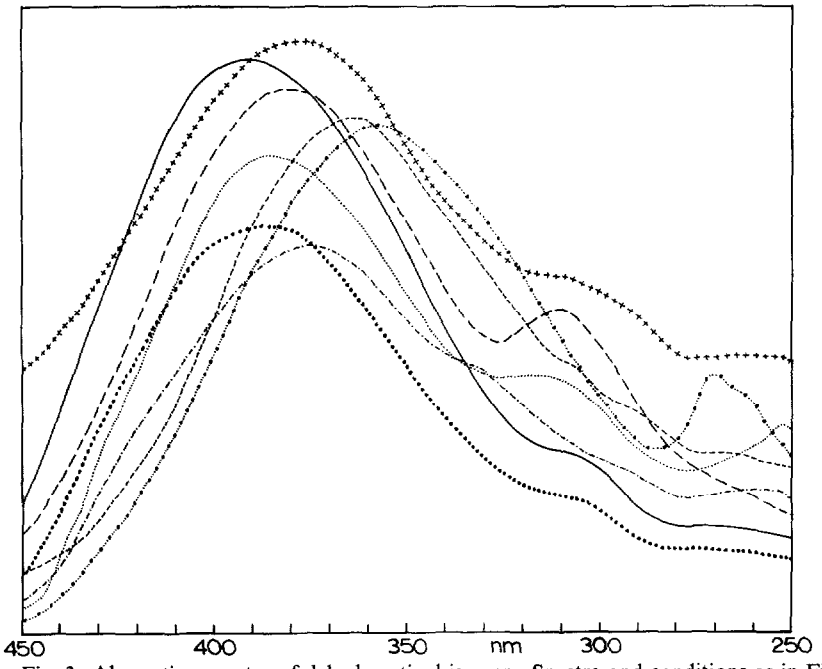

Fig. 3. Absorption spectra of dehydroretinal isomers. Spectra and conditions as in Fig. 2.

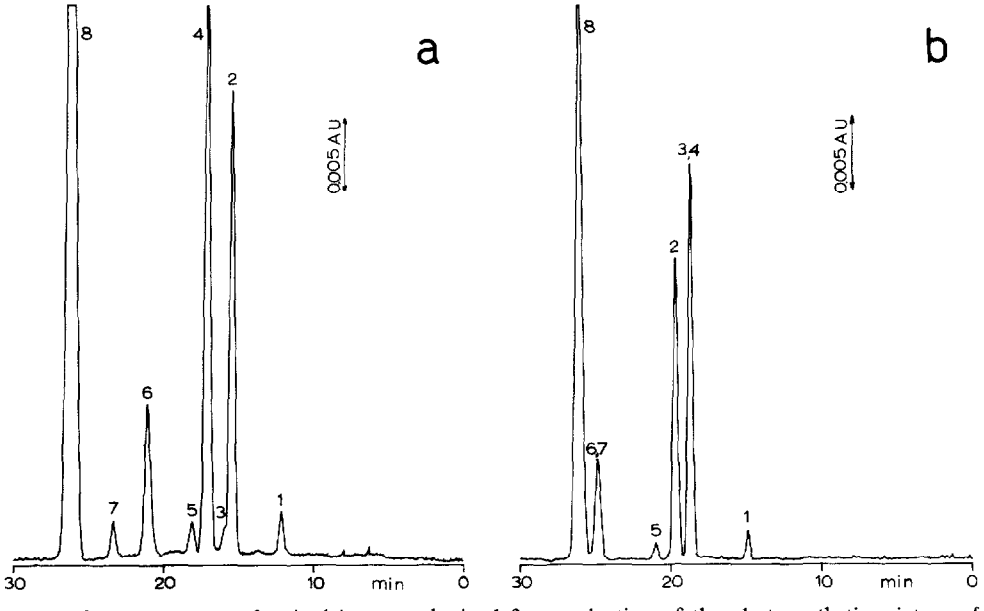

Fig. 4. Chromatograms of retinol isomers obtained from reduction of the photosynthetic mixture of retinals. Peaks: 1 = 11,13-di-*cis*; 2 = 11-*cis*; 3 = 9,13-di-*cis*; 4 = 13-*cis*; 5 = 9,11-di-*cis*; 6 = 9-*cis*; 7 = 7-*cis*; 8 = all-*trans*. Mobile phases: (a) *n*-hexane–1-octanol (96:4), flow-rate 0.6 ml/min; (b) *n*-hexane–isopropanol (99.6:0.4), flow-rate 2 ml/min. Detection wavelength, 326 nm.

retinol. A further advantage of adopting 1-octanol as a phase modifier is that for its use 4% is required instead of 0.4% (which is the optimum concentration for 2-propanol) and therefore it is easier to obtain a mobile phase that can be reproduced correctly.

It is noteworthy that when 1-octanol is used, the elution order between 11-*cis*- and 13-*cis*-retinol (Fig. 4a) is the opposite of that when 2-propanol is used (Fig. 4b), and is also the opposite of that for the corresponding aldehydes (Fig. 1a and b). This fact, seen from the relative peak heights (or areas) of 11-*cis*- and 13-*cis*-retinol (or 3-dehydroretinol), was confirmed by recording the spectra of the isomers, which are reported in Fig. 5. Together with other spectral shapes, those of 7-*cis*- and 9,11-di-*cis*-retinol are reported for the first time.

A resolution pattern and elution order similar to those of retinols were observed for 3-dehydroretinols, whose chromatograms are reported in Fig. 6a and b and were obtained by using the above two mobile phases.

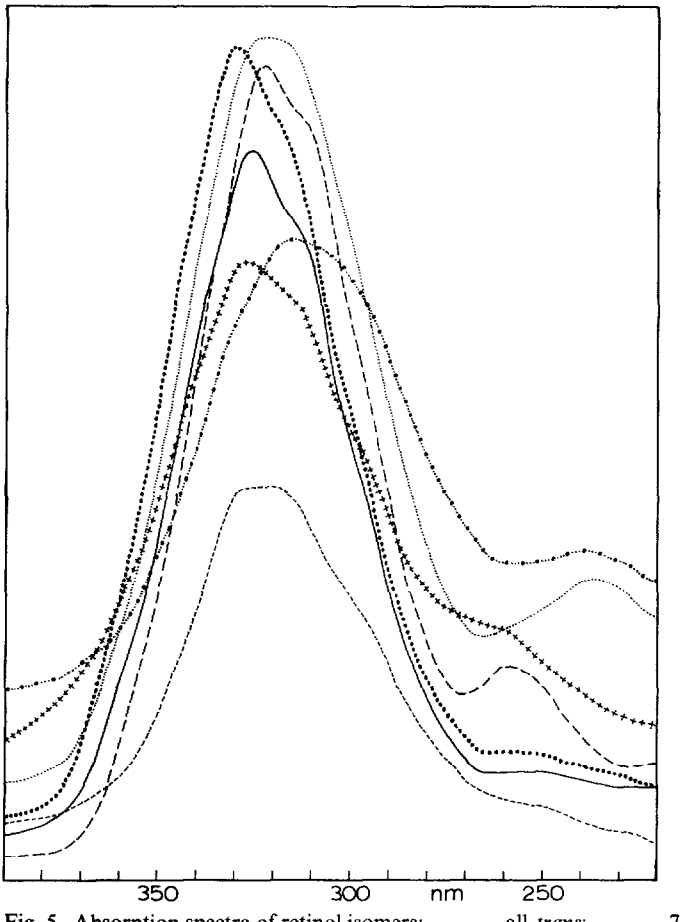

Fig. 5. Absorption spectra of retinol isomers: ———, all-*trans*; ---------, 7-*cis*; — — —, 9-*cis*; ....., 11-*cis*; ....., 13-*cis*; ●...●...●, 11,13-di-*cis*; + + +, 9,11-di-*cis*. Spectra were recorded in an 8-μl spectrophotometer cell by means of the stop-flow method. Absorbance scale in arbitrary units. Solvent: *n*-hexane–1-octanol (96:4).

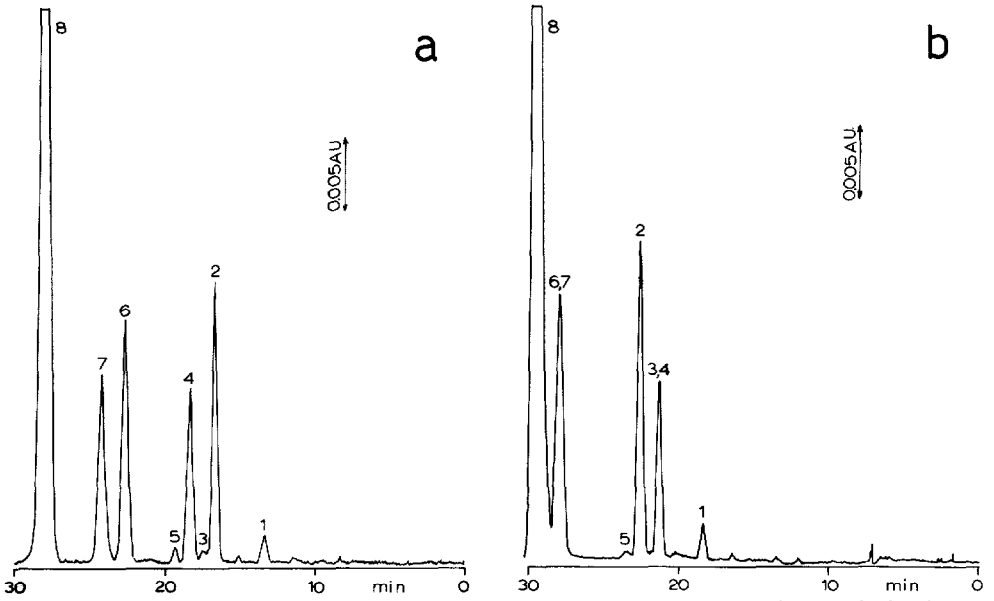

Fig. 6. Chromatograms of dehydroretinol isomers obtained from reduction of the photosynthetic mixture of dehydroretinals. Peaks and mobile phases as in Fig. 4. Detection wavelength 326 nm.

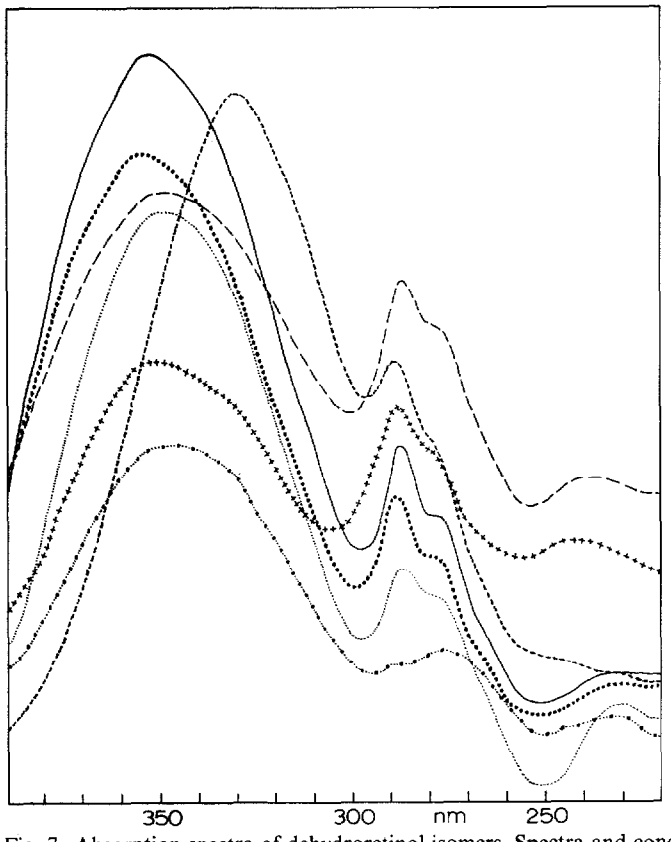

Fig. 7. Absorption spectra of dehydroretinol isomers. Spectra and conditions as in Fig. 5.

All spectra for the 3-dehydroretinols are reported in Fig. 7.

Finally, a mixture of retinols and 3-dehydroretinols was chromatographed and the simultaneous resolution of as many as thirteen peaks was achieved, as shown in Fig. 8. Such a simultaneous resolution of all major isomers of retinol and 3-dehydroretinol is most important, as it may possibly allow the most accurate definition of the vitamin A activity possessed by samples (*e.g.*, fish oils) containing both types of vitamin A compounds. In order to achieve such a goal, the calibration line of 3-dehydroretinol, the determination of its recovery from the matrix and the determination of correction factors to compensate for the different absorptivities of the different isomers at a given wavelength are still needed. Work is in progress to obtain such data.

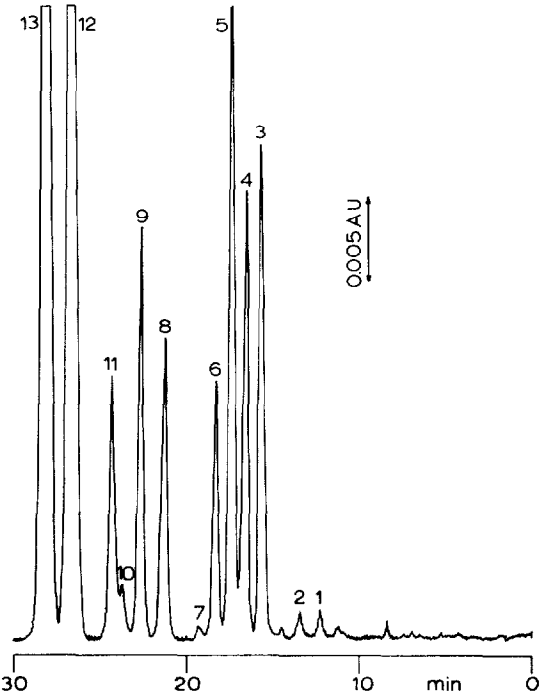

Fig. 8. Chromatogram of a mixture of retinol (A_1) and dehydroretinol (A_2) isomers. Peaks: 1 = 11,13-di-*cis*-A_1; 2 = 11,13-di-*cis*-A_2; 3 = 11-*cis*-A_1; 4 = 11-*cis*-A_2; 5 = 13-*cis*-A_1; 6 = 13-*cis*-A_2; 7 = 9,11-di-*cis*-A_2; 8 = 9-*cis*-A_1; 9 = 9-*cis*-A_2; 10 = 7-*cis*-A_1; 11 = 7-*cis*-A_2; 12 = all-*trans*-A_1; 13 = all-*trans*-A_2. Mobile phase, *n*-hexane–1-octanol (96:4); flow-rate, 0.6 ml/min; detection wavelength, 326 nm.

ACKNOWLEDGEMENT

We gratefully acknowledge the technical assistance of Sig. Franco Orel.

REFERENCES

1 B. Stancher and F. Zonta, *J. Chromatogr.*, 287 (1984) 353.
2 K. Tsukida, A. Kodama, M. Ito, M. Kawamoto and K. Takahashi, *J. Nutr. Sci. Vitaminol.*, 23 (1977) 263.

3 J. E. Paanakker and G. W. T. Groenendijk, *J. Chromatogr.*, 168 (1979) 125.
4 B. Stancher and F. Zonta, *J. Chromatogr.*, 234 (1982) 244.
5 P. V. Bhat, H. T. Co and A. Lacroix, *J. Chromatogr.*, 260 (1983) 129.
6 K. Tsukida, A. Kodama and M. Ito, *J. Chromatogr.*, 134 (1977) 331.
7 K. Tsukida, R. Masahara and M. Ito, *J. Chromatogr.*, 192 (1980) 395.
8 R. S. H. Liu and A. E. Asato, *Methods Enzymol.*, 88 (1982) 506.
9 R. S. H. Liu, A. E. Asato and M. Denny, *J. Amer. Chem. Soc.*, 99 (1977) 8095.
10 H. B. Henbest, E. R. H. Jones and T. C. Owen, *J. Chem. Soc.*, (1955) 2765.
11 R. Hubbard, P. K. Brown and D. Bownds, *Methods Enzymol.*, 18C (1971) 615.
12 R. A. S. Chandraratna, A. L. Bayerque and W. H. Okamura, *J. Amer. Chem. Soc.*, 105 (1983) 3588.
13 W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. I, Academic Press, New York, 2nd ed., 1967.