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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE UNSAPONIFIABLE FROM SAMPLES OF MARINE AND FRESHWATER FISH: FRACTIONATION AND IDENTIFICATION OF RETINOL (VITAMIN A_1) AND DEHYDRORETINOL (VITAMIN A_2) ISOMERS

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SUMMARY

Retinol (vitamin A_1) and dehydroretinol (vitamin A_2) geometric isomers, obtained in photosynthetic mixtures or from natural samples, were simultaneously fractionated by means of isocratic normal phase high-performance liquid chromatography. The use of low percentages of isopropanol (0.4–1.1%) as a modifier in hexane was studied, and the resolution of both pairs of retinols (9-*cis*/all-*trans* and 13-*cis*/11-*cis*) whose baseline separation had not previously been achieved was obtained. The proposed chromatographic method therefore allows the complete separation of all six of the most commonly occurring retinols in natural samples, including the above four mono *cis*-retinols plus the two 9,13- and 11,13-di-*cis*-retinols. Photoisomerization of retinal and of retinol in different solvent systems and during the extraction process was also studied, before analyzing the unsaponifiable from fresh and marine water fish, which constitute rich natural sources of both vitamins A_1 and A_2. Vitamin A_2 was found to be present in different relative percentages depending on the analytical matrix. Four *cis* isomers (9-*cis*-, 11-*cis*-, 13-*cis*- and 9,13-di-*cis*-retinol or corresponding dehydroretinols) were found to occur naturally together with the main all-*trans* form, confirming the need to separate geometric isomers in every dosage of vitamin A-containing compounds.

INTRODUCTION

Investigations on the biochemical properties of retinoids and their geometric isomers are presently being carried out by several workers in fields which extend from nutritional science[1] to vision research[2] and general medicine[3]; interest in the behaviour and properties of retinoids has also extended to dermatological disorders as well as to cancer research[4]. As a consequence, numerous papers have dealt with the high-performance liquid chromatography (HPLC) of geometric isomers of retinoids, (*e.g.*, retinols[5–8], retinyl esters[5,9], retinals[5,10], retinoic acid[11] and its esters[12].

Following up our two previous papers[6,7] dealing with the fractionation of retinol isomers and with the determination of total vitamin A activity in cheese, we

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report here an extension of our studies concerning the HPLC analysis of retinols. We have turned our attention to retinol isomers in order to obtain a better evaluation of vitamin A biopotency, which varies depending on the relative isomer distribution[13].

Cod-liver oil and the livers and eyes of salt- and fresh-water fish have been chosen as an analytical matrix because they constitute the richest natural sources of retinoids.

EXPERIMENTAL

Apparatus

The chromatographic apparatus consisted of a pump module (Series 3 liquid chromatograph; Perkin-Elmer, Norwalk, CT, U.S.A.), a column oven (P.E. LC 100), a variable wavelength spectrophotometric detector (P.E. LC 55 B), a digital scanner (P.E. LC 55 S) and a recorder (P.E. Model 56). Peak areas were computed by means of a chromatography data station (P.E. Sigma 15).

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

The lamp used for photolysis was a fluorescent bulb (18 W, 750 lumen, 220 V, 50 Hz) from Philips (The Netherlands).

A rotary vacuum evaporator (Rotavapor R 110; Büchi, Flawil, Switzerland) and a freeze dryer (Modulyo; Edwards, Crawley, U.K.) were also employed.

Reagents

Pure standards of vitamin A alcohol (all-*trans* retinol) and aldehyde (all-*trans* retinal), and isopropanol HPLC grade, were purchased from Fluka (Buchs, Switzerland). L-(+)-Ascorbic acid (crystalline, extra pure), potassium hydroxide (pellets, GR) and ethanol (absolute, ACS) used for alkaline digestion, as well as the hexane (HPLC grade), were purchased from Merck. Diethyl ether of ACS grade (Riedel de Haën, Hannover, F.R.G.) was used without further purification for extractions. Anhydrous sodium sulphate, dibasic sodium phosphate, monobasic potassium phosphate and sodium borohydride (analytical grade; Carlo Erba, Milan, Italy) were also employed.

Photolysis

To obtain a test mixture of retinol isomers (pure standards are not easily available), a photolytic method based on previous literature data was employed[14,15]. All-*trans* retinal was dissolved in hexane or in ethanol (about 50 mg per 100 ml). The solutions were de-aerated by bubbling nitrogen and transferred under a nitrogen stream into a spectrophotometric silica cuvette provided with a silicon rubber stopcock. The cuvette was 10 cm from the fluorescent light source. After a suitable time of exposure (the yield of the aldehyde isomer could be monitored by directly injecting aliquots of solution from the cuvette) the lamp was switched off and the aldehyde reduction was performed. The reduction process does not alter the isomer distribution[14,16]. The ethanolic solution (1 ml) was directly treated with sodium borohydride (*ca.* 5 mg) (resulting in fading of the yellow colour of the aldehydes), filtered, dried

and the residue dissolved in hexane (1 ml) for injection. After photolysis of the hexane solution, the sample must be dried and the residue dissolved in ethanol before the reduction. The procedure is then as described above. In both cases the solvent evaporation and solvent change were judged necessary because the reduction occurs in a polar solvent, whereas the injections should preferably be performed in hexane in order to avoid loss of chromatographic resolution.

Sample preparation

The sample preparation was accomplished by following a previously published procedure[17] with minor improvements. Ascorbic acid (0.5–0.7 g) as an antioxidant was weighed into a 200-ml Erlenmeyer flask, before adding the mixture for alkaline digestion, which comprised 25 ml of distilled water, 25 ml of potassium hydroxide solution (100 g per 100 ml of water) and 50 ml of ethanol. From 5 to 10 g of sample, depending on the expected vitamin A content, were weighed into the same flask after the complete dissolution of ascorbic acid, to avoid any possibility that acid catalysis could cause retinol isomerization. The flask was flushed with nitrogen and the mixture saponified overnight at room temperature. One diethyl ether extraction (200 ml of ether, shaking for 3 min at 20–25°C) was followed by washings with a phosphate buffer (according to Sörensen, pH 7.4) first employing one 100-ml aliquot, then two 50-ml aliquots. The organic extract was dried with 35 g of anhydrous sodium sulphate and evaporated by means of a rotary vacuum evaporator at 30°C. The flask containing the sample was then transferred into a vacuum freeze dryer completely to remove the residual water.

All procedures were carried out without exceeding room temperature, using amber glassware and, where possible, in a nitrogen atmosphere.

All of the sixteen possible geometric isomers of retinol have been prepared by synthesis; recently, the synthesis of the last two isomers, 7,9,11-tri-*cis* and all-*cis*, was reported by Asato *et al.*[18]. Of them, those most likely to occur in natural samples can be restricted to six, *i.e.*, all-*trans*, 13-*cis*, 11-*cis*, 9-*cis*, 9,13-di-*cis* and 11,13-di-*cis*. In the absence of pure standards, a test mixture can be obtained by means of photolysis. Retinol photolysis requires longer exposure times and does not yield the 11-*cis* isomer[14], therefore solutions of all-*trans* retinal were photolyzed in a nitrogen atmosphere followed by reduction of the isomers obtained (see Experimental). The photolysis conditions and yields were investigated in both non-polar (hexane) and polar (ethanol) solvents, previously reported to yield different distributions of products[14,15,19]. As a result, the six commonest retinol isomers were obtained. Table I summarizes their relative percentages and the rate of isomer formation with both solvent systems; in parentheses are given the values corrected in order to compensate for the different absorbances of the isomers at 326 nm. The correction factors were obtained as described under *Applications*.

11-*cis*-Retinol was formed in negligible amounts in hexane solution, while di-*cis* isomers (9,13-di-*cis*- and 11,13-di-*cis*-retinol) were formed in slightly higher percentages than in ethanol; in the latter solvent, 11-*cis*-retinol constitutes the main product of the photolysis. Fig. 1 shows the chromatographic separation of the photosynthetic mixtures obtained in hexane (a) and in ethanol (b).

TABLE I

ISOMER DISTRIBUTIONS AND RATES OF FORMATION OF RETINOLS DERIVED FROM PHOTOLYSIS OF ALL-*TRANS*-RETINAL

Values are expressed as peak area count percentages (at 326 nm) as well as percentages (in brackets) obtained by applying correction factors to peak areas (see text) in order to compensate for the different absorptions of isomers, and are referred to the total yield resulting after both photolysis and reduction processes. tr = trace.

Time (min)	*All-trans*	*9-cis*	*9,13-di-cis*	*11-cis*	*13-cis*	*11,13-di-cis*
Photolysis in hexane						
0	98.8 (98.7)	–	–	–	1.2 (1.3)	–
60	80.9 (78.3)	3.0 (3.8)	0.8 (1.0)	–	14.7 (15.6)	0.6 (1.3)
120	70.9 (67.6)	3.5 (4.4)	1.0 (1.3)	–	23.7 (24.8)	0.9 (1.9)
240	59.9 (55.0)	4.3 (5.1)	2.1 (2.6)	tr	30.4 (30.6)	3.3 (6.7)
Photolysis in ethanol						
0	98.0 (97.8)	–	–	–	2.0 (2.2)	–
60	69.1 (62.9)	3.2 (3.8)	–	12.2 (17.4)	15.1 (15.1)	0.4 (0.8)
120	56.0 (48.8)	5.6 (6.4)	0.7 (0.8)	16.6 (22.7)	20.0 (19.2)	1.1 (2.1)
240	44.7 (37.1)	7.6 (8.3)	1.3 (1.4)	21.4 (27.9)	22.4 (20.5)	2.6 (4.8)

Chromatographic system

On reviewing the literature it appeared that, among all the classes of retinoids which have been studied by means of HPLC (retinals, retinyl esters, etc.), the separation of retinols constituted a problem. Their complete fractionation had not been achieved, particularly in the case of the two pairs of retinols 9-*cis*/all-*trans* and/or 11-*cis*/13-*cis*. This problem, which was first encountered by Paanakker and Groenendijk[5], was more recently tackled by Bhat *et al.*[8] who adopted two columns: a Partisil-10-ODS and a Zorbax CN connected in series, and various 2-alkanols as mobile phase modifiers. In that study, a better resolution of one pair (9-*cis*/all-*trans*) resulted in a worse resolution of the other (11-*cis*/13-*cis*) and *vice versa*. We had already proposed normal phase HPLC as a better system for retinol resolution[6], and were able to resolve the pair 9-*cis*/all-*trans*, but the separation of the pair 11-*cis*/13-*cis* was not considered since in the retinol photosynthetic mixture used at that time 11-*cis*-retinol was absent owing to the apolar solvent employed as photolysis medium. More recently, we described the baseline separation of all-*trans*- and 9-*cis*-retinol occurring in cheese samples, which was obtained using hexane–isopropanol (99.2:0.8) as the eluent[20].

By investigating small changes in the percentage of modifier present in very low amounts (from 1.1 to 0.4%), we have been able completely to resolve all six retinols present in our mixture. A baseline separation of the two critical pairs (according to Bhat *et al.*[8]) was also achieved. Fig. 1 shows the chromatograms and chromatographic conditions. Individual isomer peaks were identified by recording their UV absorption spectra with the stop-flow method; these spectra are depicted in Fig. 2. A very slight shift of 1 nm (previously reported[6]) towards higher wavelengths due to the different solvent system used (hexane instead of ethanol) was detected in the case of some, but not all, isomers, *i.e.*, for mono-*cis* isomers but not for the two di-*cis* isomers.

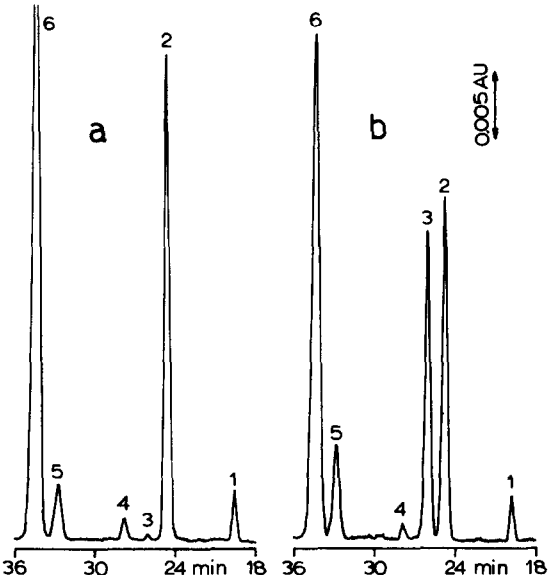

Fig. 1. Chromatograms of photosynthetic mixtures of retinol isomers in hexane (a) and in ethanol (b). Photolysis time: 4 h. For other conditions see Experimental section. Chromatographic conditions: column, Si-60, 5 μm (25 × 0.4 cm) with precolumn, pellicular silica, 40 μm (5 × 0.4 cm); mobile phase, hexane–isopropanol (99.6:0.4); flow-rate, 1.5 ml/min; temperature, 45°C; detection wavelength, 326 nm. Peaks: 1 = 11,13-di-*cis*-retinol; 2 = 13-*cis*-retinol; 3 = 11-*cis*-retinol; 4 = 9,13-di-*cis*-retinol; 5 = 9-*cis*-retinol; 6 = all-*trans*-retinol.

It is noteworthy that the elution order of retinol isomers follows that previously reported[5,16] for retinyl esters and retinals; that is, in our system, there is not the reverse order of elution between the 11-*cis*- and the 13-*cis*-retinols which was detected by Paanakker and Groenendijk[5], Groenendijk *et al.*[16] and was reported also by Bhat *et al.*[8]. This fact most probably must be ascribed to the different solvent systems used.

Noticeable changes in isomer capacity ratios (calculated by means of the formula $k'_i = t_{R_i}/t_0 - 1$, where t_{R_i} is the retention time of the *i*th peak and t_0 is the retention time of an unretained component) were observed to correspond to small changes (0.1%) in the percentages of the modifier. Fig. 3 shows the graphs obtained by plotting the capacity ratios *versus* the percentages of the modifier in the mobile phase; above 0.8% of isopropanol, the values of k' corresponding to 11-*cis*- and 13-*cis*-retinol tend to converge. Fig. 4 better illustrates the influence of a small change in isopropanol percentage on the resolution, R, of pairs of retinol isomer peaks. The resolution was calculated by means of the formula

$$R = \frac{2(t_{R_2} - t_{R_1})}{w_2 + w_1}$$

where t_{R_2} and t_{R_1} are the retention times of the components, and w_2 and w_1 are the peak widths in units of time measured at their bases. On plotting the resolution, R, of peaks corresponding to the pairs 9,13-di-*cis*/11-*cis*, all-*trans*/9-*cis* and 11-*cis*/13-*cis*

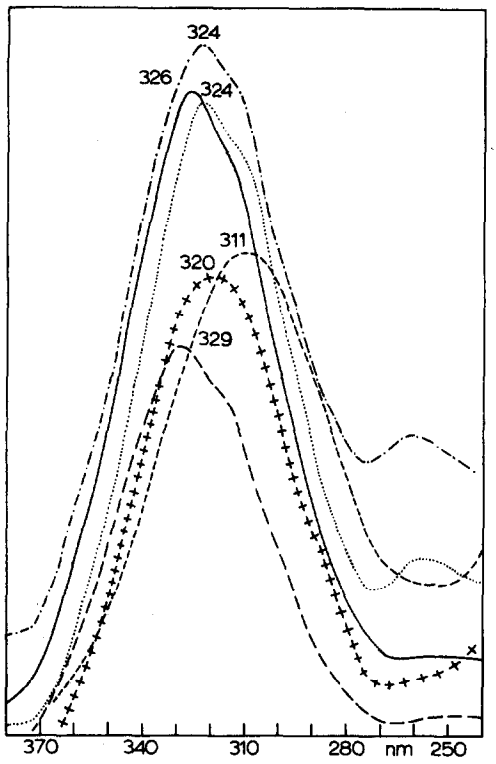

Fig. 2. Absorption spectra of retinol isomers. Spectra were recorded in the 8-μl spectrophotometer cell by means of the stop-flow method. Absorbance scale in arbitrary units. Solvent: hexane–isopropanol (99.6:0.4). ———, all-*trans*-Retinol; ....., 9-*cis*-retinol; .-.-.-, 9,13-di-*cis*-retinol; + + +, 11-*cis*-retinol; — — —, 13-*cis*-retinol; -----, 11,13-di-*cis*-retinol. The wavelength (nm) of the maximum of each spectrum is also shown.

versus the percentage of the modifier, three graphs were obtained. These gave good fits to straight lines (see dotted lines a, b, c in Fig. 4), the equations being $R = 2.40 - 0.52\%$, $R = 1.73 - 0.59\%$ and $R = 2.22 - 2.04\%$, respectively. While lines a and b follow a similar trend having a small value of the slope (which means that the change in percentage of the modifier does not appreciably alter the value of R), line c possesses a higher slope and the resolution, R, of the couple 11-*cis*/13-*cis* (to which line c refers) is strongly influenced by changes in the modifier percentage. Therefore, in our chromatographic system, only the resolution of this isomer pair is critical and requires less than 0.5–0.6% of isopropanol. The optimum resolution is obtained with 0.4% of isopropanol ($R = 1.4$) and a flow-rate of 1.5 ml/min. The analysis time can be shortened with practically no loss of resolution by setting the flow-rate at 2 ml/min.

"Blank" analysis and "spontaneous" isomerization

In this paper we are mainly concerned with naturally occurring retinoid isomers in their alcoholic forms which result from sample alkaline digestion, and therefore only retinols were given special attention. The application of retinol isomer HPLC fractionation to the analysis of vitamin A-containing samples was proposed

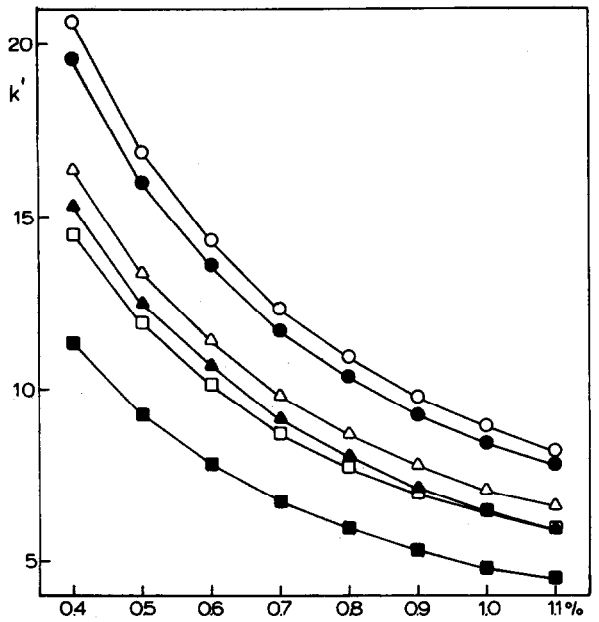

Fig. 3. Graphs of the capacity ratios, $k' = V_R'/V_0$, of retinol isomers *versus* the percentages (%) of the modifier (isopropanol) in the mobile phase (hexane). ○, all-*trans*-Retinol; ●, 9-*cis*-retinol; △, 9,13-di-*cis*-retinol; ▲, 11-*cis*-retinol; □, 13-*cis*-retinol; ■, 11,13-di-*cis*-retinol.

in order to obtain a better evaluation of vitamin activity, which is correlated with the different biopotencies of different isomers[13].

The following question arose: are isomers naturally occurring or are they derived from the extraction process or other analytical manipulations? First Groenendijk *et al.*[16] and recently others[21,22] stated that isomerization may be due to the analytical process, while Egberg *et al.*[23], Bhat *et al.*[8] and we[7,20] have reported the

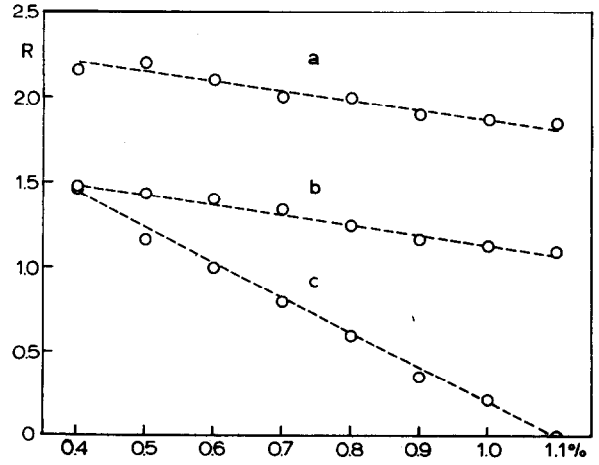

Fig. 4. Graphs of the separation coefficient, *R*, *versus* the percentages (%) of the modifier (isopropanol) in the mobile phase (hexane). Pairs: a, 9,13-di-*cis*/11-*cis*; b, all-*trans*/9-*cis*; c, 11-*cis*/13-*cis*.

natural occurrence of 13-*cis*-retinol in various samples. To answer this question, we subjected to the proposed analytical method some "blanks" of pure all-*trans*-retinol, controlling conditions which were likely to result in isomerization. A test was carried out without using amber glassware and without the special care devoted to vitamin analysis. Exposure of samples to the diffuse sunlight of the laboratory resulted in the formation of only two isomers in noticeable amounts, 9-*cis*- and 13-*cis*-retinol. No di-*cis* isomers were detected. In darkness, no 9-*cis* isomer was formed, whilst a small percentage of 13-*cis*-retinol was still detected, even when every possible precaution was adopted, *i.e.*, working at room temperature or below, in a nitrogen stream, etc. No positive evidence of a correlation of any single factor was found for the 13-*cis*-retinol formation: neither molecular oxygen (tested by bubbling air into standard solutions) nor hydrogen peroxide (added in microlitre amounts to the ether used for extraction in order to simulate the possible presence of peroxides) resulted in isomerization of all-*trans*- into 13-*cis*-retinol. A combination of factors is more likely to be the cause of the isomerization, as recently suggested by Mulry *et al.*[22] with reference to the retinyl palmitates where a correlation was found between exposure to light and the solvent used for extraction. In any case, the percentage of 13-*cis*-retinol which resulted from all the sample manipulations according to our proposed method was kept down to 1% of the all-*trans* retinol in blank tests. Therefore it appears reasonable to assume that, when the 13-*cis* isomer is found to be present in amounts far above 1% of the all-*trans* retinol (provided that all precautions have been adopted), this presence is not attributable to analytical manipulations. Some samples may have been subjected to isomerization-causing processes before analysis, as in the case of foods with long shelf lives and/or light exposure, or in the case of industrial processing. For example, cod-liver oil was found to contain, as will be

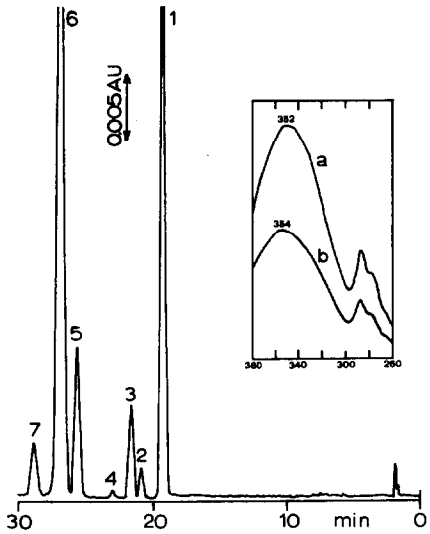

Fig. 5. Chromatogram obtained from a cod-liver oil sample. Conditions: as in Fig. 1, but flow-rate = 2 ml/min. Peaks: 1 = 13-*cis*-retinol; 2 = 13-*cis*-dehydroretinol; 3 = 9,13-di-*cis*-retinol; 4 = 9,13-di-*cis*-dehydroretinol?; 5 = 9-*cis*-retinol; 6 = all-*trans*-retinol; 7 = all-*trans*-dehydroretinol. a, Absorbance spectrum of all-*trans*-dehydroretinol (peak 7); b, absorbance spectrum of 13-*cis*-dehydroretinol (peak 2).

shown later, many retinol isomers, some of which (but not all) were also found in fish livers.

Applications

The suitability of our chromatographic system for resolving naturally occurring retinol isomers was first tested with cod-liver oil samples (which had previously been analyzed also for their vitamin D and E content[17]). The chromatogram obtained is shown in Fig. 5. When checking peak identities by means of the stop-flow method and recording of UV-absorbance spectra, spectra corresponding to dehydroretinol (vitamin A_2) isomers were also recorded. Dehydroretinol was discovered in fish as long ago as 1931 by Morton *et al.* and by Heilbron *et al.*[24], and its distribution relative to retinol varies with the species; freshwater fish contain larger amounts of dehydroretinol than marine species. Therefore, when analyzing the vitamin A content of aquatic animals (and amphibia as well) the presence of vitamin A_2 and its isomers must also be expected.

Dehydroretinol isomers follow the same elution order as the corresponding retinols: 13-*cis*-dehydroretinol is eluted shortly after 13-*cis*-retinol, and all-*trans*-dehydroretinol after all-*trans*-retinol. We subjected fish livers and eyes directly to alkaline digestion and analyzed them (see chromatograms in Figs. 6 and 7). As previously reported[24], the ratio of dehydroretinol to retinol is higher in freshwater fish than in marine fish; in all samples, retinol and/or dehydroretinol isomers other than the usual all-*trans* form were also detected, and can therefore be considered to be occurring naturally. The results are presented in Table II: the percentage distributions of retinol and dehydroretinol isomers in the samples considered are listed, assuming

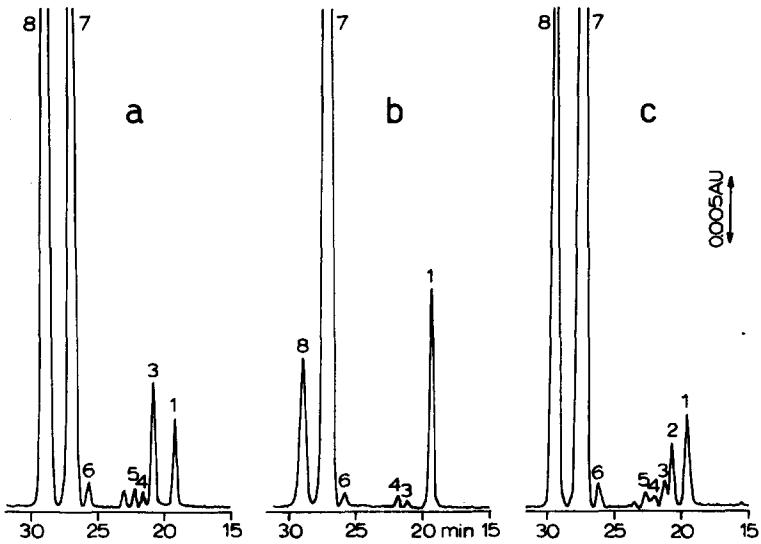

Fig. 6. Chromatograms obtained from the unsaponifiable of fish livers: a, rainbow trout; b, eel; c, pilchard sardine. Conditions as in Fig. 5. Peaks: 1 = 13-*cis*-retinol; 2 = 11-*cis*-retinol; 3 = 13-*cis*-dehydroretinol; 4 = 9,13-di-*cis*-retinol; 5 = 11-*cis*-dehydroretinol?; 6 = 9-*cis*-retinol; 7 = all-*trans*-retinol; 8 = all-*trans*-dehydroretinol.

TABLE II

RETINOL AND DEHYDRORETINOL ISOMER DISTRIBUTIONS IN SOME SAMPLES FROM MARINE AND FRESHWATER FISH

Values are reported as percentages of total retinols and dehydroretinols, and were computed by applying correction factors (see text) to peak areas, in order to compensate for different absorptions as recorded at 326 nm. tr. = trace.

Sample	*Retinol isomers*					*Dehydroretinol isomers*				
	all-trans	*9-cis*	*9,13-di-cis*	*11-cis*	*13-cis*	*all-trans*	*9-cis*	*9,13-di-cis*	*11-cis*	*13-cis*
Cod (*Gadus morhua*, Linnaeus) liver oil	48.8	9.8	5.9	–	27.5	4.8	–	tr.	–	3.1
Rainbow trout (*Salmo gairdneri irideus*, Richardson) liver	27.2	1.0	tr.	–	1.9	65.0	–	–	tr.	4.9
Eel (*Anguilla anguilla*, Linnaeus) liver	89.0	0.5	tr.	–	4.2	5.9	–	–	–	0.4
Pilchard sardine (*Clupea pilchardus pilchardus*, Walbaum) liver	62.1	1.3	tr.	2.3	2.3	30.5	–	–	tr.	1.5
Pilchard sardine eyes	70.3	3.1	1.7	14.1	10.8	–	–	–	–	–

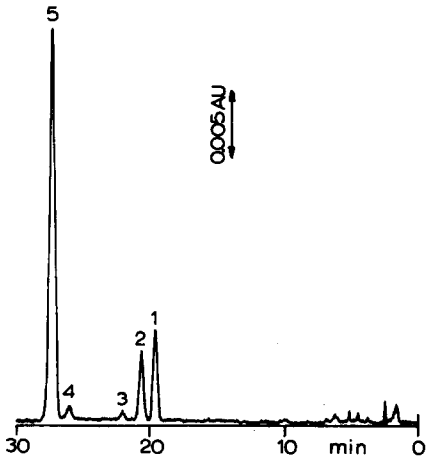

Fig. 7. Chromatogram obtained from the unsaponifiable of pilchard sardine eyes. Conditions as in Fig. 5. Peaks: 1 = 13-*cis*-retinol; 2 = 11-*cis*-retinol; 3 = 9,13-di-*cis*-retinol; 4 = 9-*cis*-retinol; 5 = all-*trans*-retinol.

that recovery from the matrix is the same for all compounds. All peak areas were computed at 326 nm. Since at this wavelength every retinol and dehydroretinol isomer possesses a different extinction, correction factors were calculated by dividing the absorbance of all-*trans*-retinol by that of every other isomer at 326 nm, as extrapolated from literature data[13,25]. The factors obtained are as follows: all-*trans*-retinol, 1; 13-*cis*-retinol, 1.10; 9-*cis*-retinol, 1.31; 9,13-di-*cis*-retinol, 1.34; 11-*cis*-retinol, 1.57; 11,13-di-*cis*-retinol, 2.21; all-*trans*-dehydroretinol, 1.64; 13-*cis*-dehydroretinol, 1.78; those may be used as good approximations when pure standards are lacking. The use of the correction factors is essential as values expressed simply as area percentages may greatly distort the evaluation of the real distributions of both retinol and dehydroretinol and consequently the assessment of total vitamin A biopotency. Obviously this is more important for the isomers with the highest correction factors.

Cod-liver oil was found to contain more vitamin A isomers than other unprocessed samples. This may derive from the extraction or processing method employed as well as from improper storage or long shelf life. The HPLC method appears suitable for testing the quality of cod-liver oil as an accurate determination of the vitamin A biopotency is obtainable by a critical evaluation of the isomer distribution.

Approximate ratios between retinol and dehydroretinol of 2:1 and 1:2 respectively in marine (pilchard sardine liver) and freshwater (rainbow trout liver) samples were found. The presence of 11-*cis*-retinol (confirmed spectroscopically) in fish liver was hardly expected, in contrast to eyes where it plays a natural rôle.

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