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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINING THE ISOMER DISTRIBUTION OF RETINOL (VITAMIN A_1) AND 3-DEHYDRORETINOL (VITAMIN A_2) IN FISH OILS

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SUMMARY

Seven retinol and seven 3-dehydroretinol geometric isomers were simultaneously fractionated on a silica column using a ternary mobile phase (2-propanol–1-octanol–*n*-hexane, 0.2:3.8:96). The method was applied to determine vitamin A active compounds present in fish oils. The standard additions method was used to obtain the recovery of the analytes, which was found to be the same for vitamin A_1 and A_2 (89%). Correction factors for determining all isomers when the peak areas are integrated at 326 nm are reported, so that the quantitative determination may be reproduced by others using the proposed method, which allows all data to be referred to the calibration graph of all-*trans*-retinol only. As every isomer is quantified separately, the method also permits the determination of the real vitamin A biopotency of the samples.

INTRODUCTION

The separation of retinols and 3-dehydroretinols and their relative distribution in fish samples, as obtained by means of high-performance liquid chromatography (HPLC), was recently described[1]. The chromatographic system was successively improved by changing the mobile phase composition, which resulted in the separation (from a photosynthetic isomer mixture) of as many as thirteen vitamin A isomers[2].

In this paper we describe the use of the ternary eluent mixture 1-octanol–2-propanol–*n*-hexane, which permits the separation of all of the fourteen most commonly occurring vitamin A_1 and A_2 isomers. The main purpose of this paper is to present a solution to the problems connected with the quantitative determination of the isomers.

After having synthesized pure all-*trans*-3-dehydroretinol[2], we have now constructed its calibration graph and correlated the slope obtained with that of the all-*trans*-retinol calibration graph. Such a correlation results in the avoidance of any inconvenience connected with the unavailability of all-*trans*-3-dehydroretinol. The correction factors[1] that must be applied to all detected retinols and 3-dehydroretinols in order to compensate for their different absorbances at the detection wavelength

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of 326 nm (maximum for all-*trans*-retinol) have been confirmed, and others have been added. The recoveries of both vitamin A_1 and A_2 were determined by ratioing the slopes of the standard additions graphs with those of the calibration graphs.

The method presented here is suitable for ready application by other laboratories, where the same analysis could be performed after having determined the calibration graph of all-*trans*-retinol only. Further, by determining each isomer separately, the final evaluation of the total vitamin A biopotency should result in close agreement with values otherwise obtainable only by using biological methods, which are complicated and much more time consuming.

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) and a recorder (Perkin-Elmer Model 56). A Chromatopac C-R 1 B data processor (Shimadzu, Kyoto, Japan) was used as the integrator.

A guard column (5 × 0.4 cm I.D.) (Supelco, Bellefonte, PA, U.S.A.), dry packed with a 40-μm silica pellicular packing, was always used, 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 employed.

The lamp used for the photolysis was a fluorescent bulb (18 W, 750 lumen) from Philips (Eindhoven, The Netherlands).

A rotary vacuum evaporator (Rotavapor R 110; Büchi, Flawil, Switzerland), a freeze dryer (Modulyo; Edwards, Crawley, U.K.) and a UV–visible spectrophotometer (Super Scan Model 3; Varian Techtron, Springvale, Australia) were also employed.

Reagents

2-Propanol (HPLC grade), L-(+)-ascorbic acid (crystalline, extra pure) and potassium hydroxide (pellets, reagent grade) were purchased from Merck.

n-Hexane (HPLC grade), anhydrous sodium sulphate, dibasic sodium phosphate, monobasic potassium phosphate and sodium borohydride (analytical-reagent grade) were purchased from Carlo Erba (Milan, Italy).

1-Octanol (reagent grade), ethanol (absolute, ACS) and diethyl ether (ACS) were from Riedel de Haën (Hannover, F.R.G.).

Pure standards of vitamin A aldehyde (all-*trans*-retinal) and vitamin A alcohol (all-*trans*-retinol) were obtained from Fluka (Buchs, Switzerland).

Vitamin A_2 aldehyde (3-dehydroretinal) was prepared by synthesis and the pure all-*trans* form was obtained by semi-preparative HPLC as previously described[2]. All-*trans*-3-dehydroretinol was obtained by sodium borohydride reduction of the aldehyde[2].

Methods

The sample preparation was slightly modified from the previously published procedure[1], as far as the volumes of reagents involved are concerned. Into an amber-coloured 100-ml flask was weighed 0.1 g of ascorbic acid and 5 ml of distilled water, 10 ml of ethanol and 5 ml of potassium hydroxide solution (100 g per 100 ml) were added. Then, 1.00 g of fish oil was added and the flask was flushed with nitrogen, fitted with a stopcock and alkaline digestion was carried out overnight at room temperature with magnetic stirring. The sample was extracted with one 50-ml aliquot of diethyl ether, and the organic phase was washed with 20 ml of phosphate buffer (pH 7.7) and twice more with 10-ml aliquots. A 10-g amount of anhydrous sodium sulphate was added to the separating funnel and the extract was then filtered (on paper; 589[1] Schwarzband, Schleicher & Schüll, Dassel, F.R.G.) into a 50-ml flask and evaporated by means of a rotary vacuum evaporator. To remove any remaining traces of water, the sample was further dried in a freeze dryer. The remaining oil was quantitatively transferred, by using the chromatographic mobile phase as solvent, into a 5-ml volumetric flask. Volumes of 10 μl of this sample solution were used for the injections. To obtain the amount of the required analyte present in 1.00 g of the sample, the amount found in the volume injected (10 μl) must be multiplied for 500 (as 1.00 g of sample was dissolved in a final volume of 5 ml).

For constructing the calibration graphs and for obtaining the recoveries by means of the standard additions method, solutions of all-*trans*-retinol and all-*trans*-3-dehydroretinol were prepared. Two different retinol solutions (with the same concentration, 10 mg per 100 ml) were prepared, in 1-octanol–*n*-hexane (4:96) and in ethanol. We observed that vitamin A is much more soluble in ethanol than in *n*-hexane; further, in *n*-hexane the vitamin decomposition occurs faster than in alcoholic solutions. The concentrations of the two solutions were determined by measuring their absorbance by means of a UV–visible spectrophotometer [after suitable dilution (1:25)] using 1 cm cells. The absorbance values obtained were consistent with those calculated applying the absorptivities ($A_1^{1\%}{}_{cm}$) taken from the literature[3]. The 1-octanol–*n*-hexane solution was used for constructing the calibration graph (in the range from 0 to 1.2 μg) by directly injecting microlitre aliquots into the chromatograph. The ethanol solution was used for the standard additions, adding 1.00, 2.00 and 3.00 ml to three separate samples of the same oil, which were then submitted to alkaline digestion.

All-*trans*-3-dehydroretinol was first dissolved (10 mg per 100 ml) in *n*-hexane–1-octanol (96:4), giving a stock solution that was then diluted 10-fold. This working solution (1 mg per 100 ml) was used to obtain the calibration graph for all-*trans*-3-dehydroretinol (in the range from 0 to 0.24 μg) by directly injecting microlitre aliquots into the chromatograph. The solution of all-*trans*-3-dehydroretinol to be used for the standard additions was prepared in ethanol (10 mg per 100 ml) and then diluted 5-fold (2 mg per 100 ml) before being employed for the additions, as described above for all-*trans*-retinol.

Data processing

Statistical analysis of data was performed with an Olivetti P6040 desk calculator. The linearity of the calibration graphs and of the standard addition graphs was tested by the analysis of the variance (ANOVA) for the regression, using R^2 and F

ratios as criteria of adequacy[4]. R^2 is the ratio between the sum of squares attributable to regression and the total sum of squares, and F is the ratio between the variance attributable to regression and the variance attributable to deviation from regression. s_{xy} is the standard deviation of the regression.

Oil samples

Fish oil samples used for testing the proposed analytical method were kindly provided by Professor R. G. Ackman, Canadian Institute of Fisheries Technology, Technical University of Nova Scotia, Halifax, Canada. Oil samples were numbered as follows.

Sample No. 1. Yarmouth cod (*Gadus morhua*, Linnaeus) liver oil, extracted with dichloromethane by grinding with sodium sulphate, mixed fish, length 39–51 cm.

Sample No. 2. Yarmouth cod liver oil, extracted with dichloromethane by grinding with sodium sulphate, fish caught at Brown's Bank, August 1983, length 59 cm, weight 1.750 g, liver 23.3 g, 43.1% (w/w) oil.

Sample No. 3. Retail cod liver oil, Pharmo Brand RGA-LX-118-D, Lot (L) 4958, expiry date October 1984, label vitamin A concentration 3900 IU per 5 ml.

Sample No. 4. Redfish (*Sebastes marinus*, Linnaeus) oil, body and viscera, Canso Fisheries Reduction Plant, January 17th, 1984, RGA-IX-115-E.

RESULTS AND DISCUSSION

Chromatographic conditions

When 1-octanol was used as a phase modifier in *n*-hexane for the chromatographic resolution of a photosynthetic mixture of retinol and 3-dehydroretinol isomers, a satisfactory separation of thirteen peaks was achieved[2]. Fig. 1 shows the chromatogram. The shaded peak (numbered 6, 7) is a composite one, consisting mainly of 13-*cis*-3-dehydroretinol, but containing a small unresolved amount of 9,11-di-*cis*-retinol. Previously, little attention was paid to this peak, as the photolytic process leads to a small amount only of 9,11-di-*cis*-retinol (see peak 7 in Fig. 2).

On performing the same chromatographic separation with samples from the unsaponifiable matter of fish oils, the relative percentages of the two isomers were different, and the main component was shown to be 9,11-di-*cis*-retinol instead of 13-*cis*-3-dehydroretinol. The peak was confirmed to be a composite one by recording its absorption spectrum, which showed a mixed profile of the two components. Hence the necessity for resolving such a peak arose.

We had already noticed that 1-octanol had the effect of resolving better the peaks with higher retention times, whereas 2-propanol was effective in separating the early eluting peaks; we concluded that a ternary mixture of solvents should therefore lead to the resolution of all fourteen of the most commonly occurring isomers. A suitable mobile phase was found, consisting of 3.8% of 1-octanol and 0.2% of 2-propanol in *n*-hexane, and the chromatogram obtained is shown in Fig. 2. Higher percentages of 2-propanol result in a decrease in resolution among the 9-*cis*, 7-*cis* and all-*trans*-isomers, while the group of 11-*cis* and 13-*cis* isomers shows a tendency for overlapping. By using 2-propanol alone, the elution order between the 11-*cis* and 13-*cis* isomers is reversed[2]. The result presented is the best compromise because, while

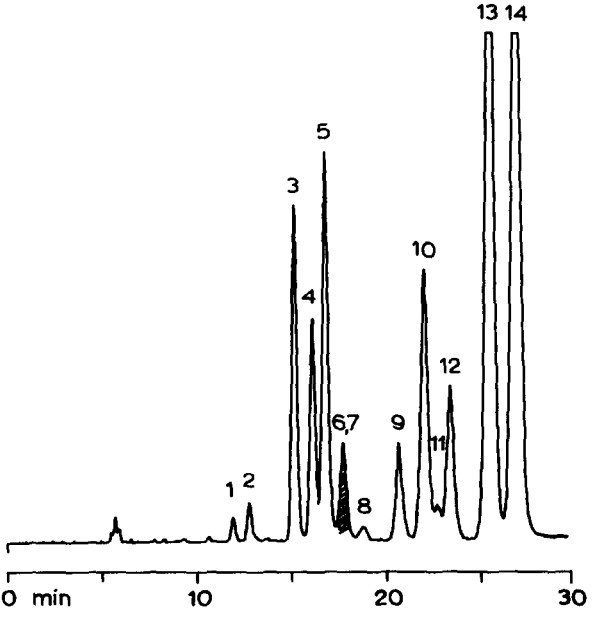

Fig. 1. Chromatogram of the photosynthetic mixture of retinol (A_1) and 3-dehydroretinol (A_2) isomers. Peaks: 1 = 11,13-di-*cis*-A_1; 2 = 11,13-di-*cis*-A_2; 3 = 11-*cis*-A_1; 4 = 11-*cis*-A_2; 5 = 13-*cis*-A_1; 6 = 13-*cis*-A_2; 7 = 9,11-di-*cis*-A_1; 8 = 9,11-di-*cis*-A_2; 9 = 9-*cis*-A_1; 10 = 9-*cis*-A_2; 11 = 7-*cis*-A_1; 12 = 7-*cis*-A_2; 13 = all-*trans*-A_1; 14 = all-*trans*-A_2. Mobile phase: *n*-hexane–1-octanol (96.1:3.9); flow-rate, 0.6 ml/min; detection wavelength, 326 nm.

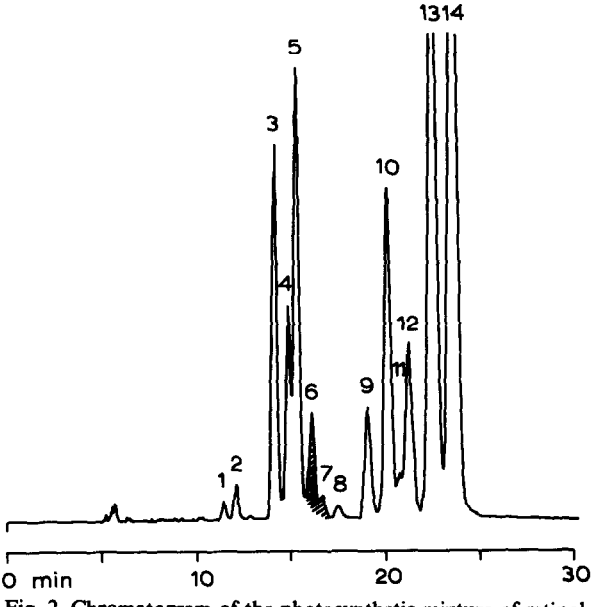

Fig. 2. Chromatogram of the photosynthetic mixture of retinol (A_1) and 3-dehydroretinol (A_2) isomers. Peaks as in Fig. 1. Mobile phase: *n*-hexane–1-octanol–2-propanol (96:3.8:0.2); flow-rate, 0.6 ml/min; detection wavelength, 326 nm.

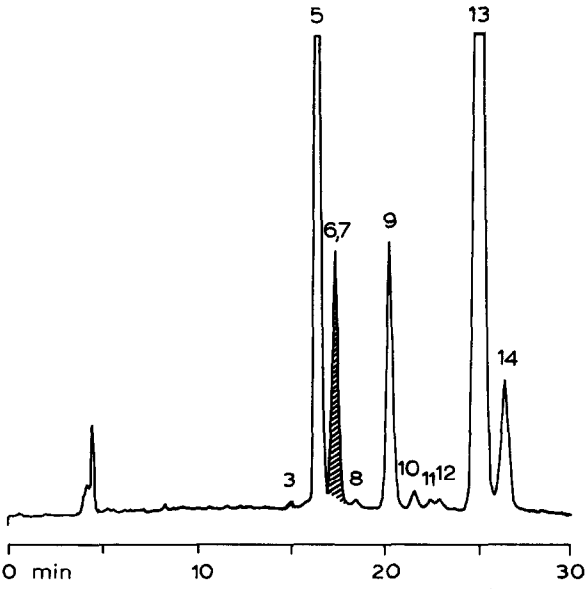

Fig. 3. Chromatogram of the retinol and 3-dehydroretinol isomers from cod liver oil (sample No. 1). Peaks and conditions as in Fig. 1.

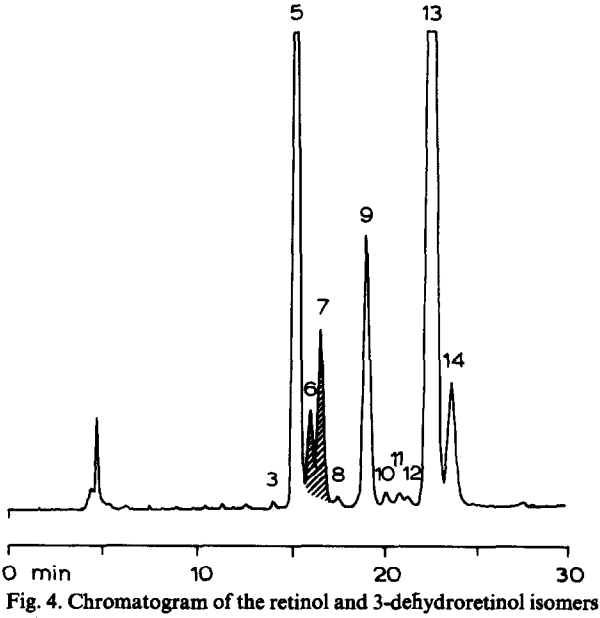

Fig. 4. Chromatogram of the retinol and 3-dehydroretinol isomers from cod liver oil (sample No. 1). Peaks and conditions as in Fig. 2.

resolving all fourteen peaks, the decrease in resolution between the pair of peaks 4 and 5 (see Figs. 1 and 2) is minimal. The analysis time with the new ternary mobile phase is shortened by more than 15%.

Figs. 3 and 4 show the chromatograms obtained from fish oil (sample No. 1) with the binary and the ternary mobile phase, respectively.

The improved separation of the shaded peaks 6 and 7 is most evident in Fig. 4. The decrease in resolution of the pair of peaks 4 and 5 is irrelevant with the oil samples considered, which do not contain 11-*cis*-3-dehydroretinol.

Quantitative analysis

All peaks were detected and integrated at 326 nm, which is the wavelength of the maximum absorbance of all-*trans*-retinol, chosen as the reference compound. As at this wavelength every other retinol and 3-dehydroretinol possesses a different absorptivity, correction factors for all other peak areas must be used[1]. These factors (f_i) may be calculated either by dividing the absorbance of all-*trans*-retinol by that of every other isomer at 326 nm, as extrapolated from literature data[3,5], or by applying the equation

$$f_i = \frac{A_{1\,\text{cm}}^{1\%}\,(\lambda_{max}=326)\ (\text{all-}trans\text{-retinol})}{A_{1\,\text{cm}}^{1\%}\,(\lambda_{max}\ \text{of}\ i)\ (\text{isomer}\ i)} \cdot \frac{\text{Peak area}\ i\ (\lambda_{max}\ \text{of}\ i)}{\text{Peak area}\ i\ (\lambda_{max} = 326)}$$

Both methods were used when possible, and the mean values obtained are reported in Table I. It was not possible for us to calculate the f_i relative to 7-*cis*-retinol and 7-*cis*-3-dehydroretinol, because the values of $A_{1\,\text{cm}}^{1\%}$ of such isomers could not be found in the literature. As such isomers were found to be present only in trace amounts, this fact is irrelevant in determining the total vitamin A content of the considered samples.

Fig. 5 shows the calibration graph and the graphs obtained by means of the standard additions method for all-*trans*-retinol (vitamin A_1), and Fig. 6 shows the corresponding graphs for all-*trans*-3-dehydroretinol (vitamin A_2).

The parameters of all graphs are given in Table II. The amounts (Q) of the analytes (all-trans-A_1 and all-*trans*-A_2) present in the samples are given by the in-

TABLE I

CORRECTION FACTORS FOR RETINOL AND 3-DEHYDRORETINOL ISOMERS

Isomer peak areas (when recorded at 326 nm) must be multiplied by the listed factors in order to correlate all areas to that of all-*trans*-retinol.

Isomer	*Retinol isomers*	*3-Dehydroretinol isomers*
All-*trans*	1.00	1.65
13-*cis*	1.10	1.76
11-*cis*	1.56	2.20
9-*cis*	1.31	1.95
11,13-Di-*cis*	2.21	2.22
9,11-Di-*cis*	1.33	2.25

TABLE II

PARAMETERS OF THE CALIBRATION GRAPHS AND OF THE STANDARD ADDITION GRAPHS OF ALL-*TRANS*-RETINOL AND ALL-*TRANS*-3-DEHYDRORETINOL

The values of the standard addition graphs refer to the four different fish oils considered. As $y = a + bx$ is the general equation of the graphs, a and b represent the intercept and the slope respectively, s_a and s_b being their standard deviations; s_{xy} is the standard deviation of the regression. Q, defined by the absolute value of the ratio $-a/b$, represents the amount of the analyte contained in the volume (10 μl) of the sample injected, s_Q being the standard deviation. Recoveries, P, were obtained dividing the b values of the standard additions graphs by those of the calibration graphs, s_P being their standard deviation.

Graph	*Compound*	a	s_a	b	s_b	s_{yx}	F	R^2	$Q \pm s_Q$	$P \pm s_P$
Calibration	All-*trans*-retinol	$-0.005 \cdot 10^6$	$0.007 \cdot 10^6$	$1.724 \cdot 10^6$	$0.009 \cdot 10^6$	$0.008 \cdot 10^6$	32,781	0.9999		
	All-*trans*-3-dehydroretinol	$0.001 \cdot 10^6$	$0.002 \cdot 10^6$	$1.045 \cdot 10^6$	$0.010 \cdot 10^6$	$0.002 \cdot 10^6$	11,568	0.9998		
Standard additions	Sample 1									
	All-*trans*-retinol	$0.782 \cdot 10^6$	$0.011 \cdot 10^6$	$1.517 \cdot 10^6$	$0.031 \cdot 10^6$	$0.014 \cdot 10^6$	2455	0.9992	0.515 ± 0.013	88.5 ± 1.8
	All-*trans*-3-dehydroretinol	$0.078 \cdot 10^6$	$0.001 \cdot 10^6$	$0.930 \cdot 10^6$	$0.018 \cdot 10^6$	$0.002 \cdot 10^6$	2797	0.9993	0.087 ± 0.002	88.9 ± 1.9
	Sample 2									
	All-*trans*-retinol	$0.582 \cdot 10^6$	$0.003 \cdot 10^6$	$1.537 \cdot 10^6$	$0.008 \cdot 10^6$	$0.004 \cdot 10^6$	33,126	0.9999	0.379 ± 0.003	89.1 ± 0.7
	All-*trans*-3-dehydroretinol	$0.102 \cdot 10^6$	$0.002 \cdot 10^6$	$0.927 \cdot 10^6$	$0.027 \cdot 10^6$	$0.002 \cdot 10^6$	1174	0.9983	0.110 ± 0.004	88.7 ± 2.7
	Sample 3									
	All-*trans*-retinol	$0.651 \cdot 10^6$	$0.010 \cdot 10^6$	$1.532 \cdot 10^6$	$0.028 \cdot 10^6$	$0.012 \cdot 10^6$	3053	0.9993	0.425 ± 0.010	88.9 ± 1.7
	All-*trans*-3-dehydroretinol	$0.021 \cdot 10^6$	$0.002 \cdot 10^6$	$0.914 \cdot 10^6$	$0.022 \cdot 10^6$	$0.002 \cdot 10^6$	1654	0.9994	0.023 ± 0.002	87.5 ± 2.3
	Sample 4									
	All-*trans*-retinol	$0.496 \cdot 10^6$	$0.005 \cdot 10^6$	$1.551 \cdot 10^6$	$0.014 \cdot 10^6$	$0.006 \cdot 10^6$	11,921	0.9998	0.320 ± 0.004	90.0 ± 0.9
	All-*trans*-3-dehydroretinol	$0.026 \cdot 10^6$	$0.001 \cdot 10^6$	$0.948 \cdot 10^6$	$0.017 \cdot 10^6$	$0.001 \cdot 10^6$	3168	0.9993	0.027 ± 0.001	90.7 ± 1.8

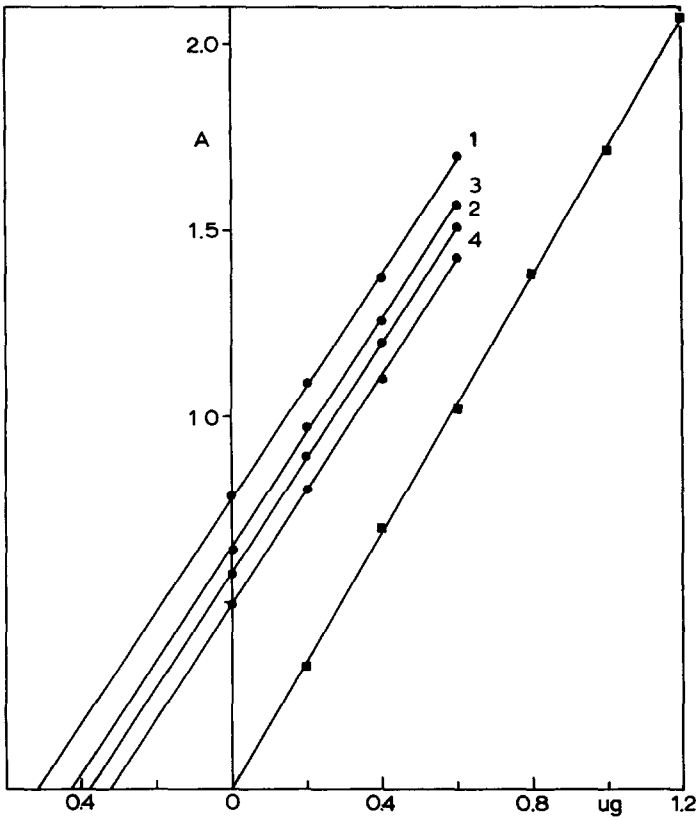

Fig. 5. Calibration graph for all-*trans*-retinol (■) and graphs obtained by means of the standard additions method (●) for all-*trans*-retinol for samples 1, 2, 3 and 4. The graphs were obtained by plotting the absolute amount (μg, abscissa) of the analyte *versus* the area count (ordinate). The values of the area count must be multiplied by 10^6 to obtain the values reported by the integrator used.

tercepts of the standard addition graphs ($y = a + bx$) with the abscissa, and can therefore be obtained by calculating the absolute value of the ratio $-a/b$. The percentage recoveries (P) of the analytes were obtained by dividing the slope of the standard addition graphs by the slope of the calibration graphs and multiplying by 100. Such values are virtually the same for both vitamins (all-*trans*-A_1 and -A_2), the mean values for the four analysed samples being 89.1 ± 0.6 (A_1) and 89.0 ± 1.32 (A_2), respectively. A recovery of 89.0% may therefore be used for both vitamins with very close approximation, because the two mean values are not significantly different after considering their standard deviations.

We further consider acceptable the assumption that the recovery is the same for all other isomers.

The standard additions method requires several (at least four) replicate analyses of the same sample. It is possible to carry out a single analysis per sample if the recovery of the analyte (from a given matrix) is known and its calibration graph has been constructed.

TABLE III

DISTRIBUTION OF VITAMIN A ISOMERS IN FISH OILS

All values are expressed as μg/g, and were calculated after correcting for the common recovery (89%) and for the different absorptivities of the isomers at 326 nm (applying the correction factors reported in Table I). Values in parentheses are those attributable to the biopotency of the various isomers[3]. Values ≤1 μg/g were considered as trace amounts (tr.), because they represent less than about 0.5% of the total amount, although the spectrophotometric detection limits, combined with larger injection volumes, would permit the determination of smaller amounts of the analytes.

Sample No.	*13-cis-Retinol (biopotency, 75%)*	*9,11-Di-cis-retinol (biopotency, x%)*	*9-cis-Retinol (biopotency, 22%)*	*All-trans-retinol (biopotency, 100%)*	*13-cis-3-Dehydroretinol (biopotency, 35%)*	*9,11-Di-cis-3-dehydroretinol (biopotency, y%)*	*9-cis-3-Dehydroretinol (biopotency, 14%)*	*All-trans-3-dehydroretinol (biopotency, 51%)*	*Total, retinols + 3-dehydroretinols*	*Total biopotency A*
1	135.9 (101.9)	30.5	57.3 (12.6)	257.5	23.9 (8.4)	8.4	8.8 (1.2)	43.5 (22.2)	565.8	(403.8 + 30.5*x* + 8.4*y*)
2	8.8 (6.6)	3.7	7.5 (1.6)	189.5	tr.	tr.	4.7 (0.7)	55.0 (28.1)	269.2	(226.5 + 3.7*x*)
3	52.7 (39 5)	11.0	21.5 (4.7)	212.5	8.1 (2.8)	tr.	tr.	11.5 (5.9)	317.3	(265.4 + 11.0*x*)
4	33.5 (25.1)	6.2	11.2 (2.5)	160.0	6.0 (2.1)	tr.	tr.	13.5 (6.9)	230.4	(196.6 + 6.2*x*)

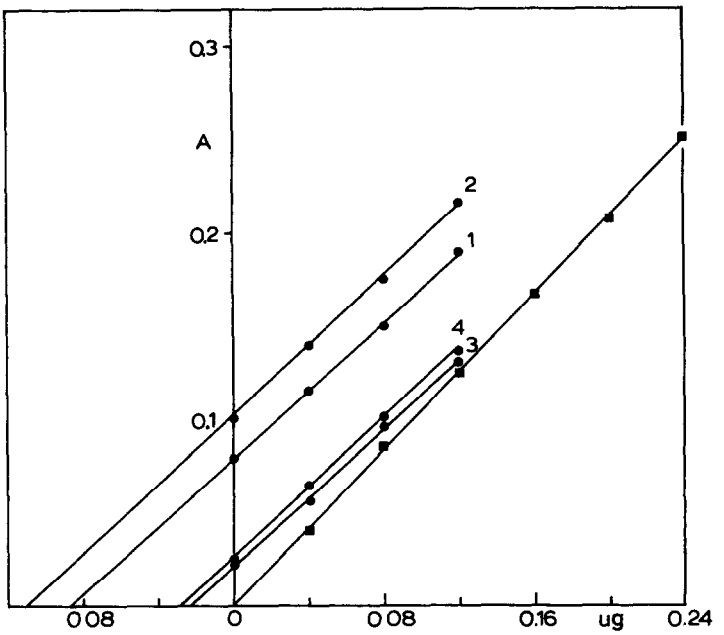

Fig. 6. Calibration graph for all-*trans*-3-dehydroretinol (■) and graphs obtained by means of the standard additions method (●) for all-*trans*-3-dehydroretinol for samples 1, 2, 3 and 4. Abscissa and ordinate as in Fig. 5.

As vitamin A_2 (all-*trans*-3-dehydroretinol) is not commercially available, we synthesized and purified it[2] and used it to determine its recovery and calibration graph. The slope of the all-*trans*-3-dehydroretinol calibration graph ($b_2 = 1.045 \cdot 10^6$) can be correlated with that of all-*trans*-retinol ($b_1 = 1.724 \cdot 10^6$). The correction factor ($f = b_1/b_2 = 1.65$) is consistent with that previously obtained (1.64) by extrapolating the absorbance from literature data[1]. Therefore, after having defined all the analytical conditions, only one analysis per sample can be performed, using the obtained recovery and the correction factors listed in Table I for the isomers, and referring all values to the vitamin A_1 calibration graph.

Table III reports the contents of retinol and dehydroretinol isomers in four different oil samples. The vitamin A biopotency[3] values are also reported in parentheses, excluding the 9,11-di-*cis* isomers, for which biopotency data were not available. It can be seen that the total biopotencies obtained are different from those of the total amounts; hence, in to our opinion, the quantitative determination of each isomer by HPLC is essential in every vitamin A analysis.

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