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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FAT-SOLUBLE VITAMINS

SIMULTANEOUS QUANTITATIVE ANALYSIS OF VITAMINS D_2, D_3 AND E. STUDY OF PERCENTAGE RECOVERIES OF VITAMINS FROM COD LIVER OIL

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SUMMARY

Vitamins D_2, D_3 and E were resolved and quantified by applying reversed-phase high-performance liquid chromatography to extracts of cod liver oil. The method, using two reversed-phase C_{18} columns and a ternary mixture of acetonitrile, methanol and water as the eluent resolved all fat-soluble vitamins well, including the pair D_2–D_3. The extraction procedure was studied; the recoveries, using two different solvents (hexane and diethyl ether) for extractions were 60.6 $\pm$ 1.0 and 77.1 $\pm$ 1.1, 56.9 $\pm$ 1.2 and 74.8 $\pm$ 0.8, and 14.1 $\pm$ 0.7 and 89.8 $\pm$ 1.4 % for vitamins D_2, D_3 and E, respectively.

INTRODUCTION

In the application of high-performance liquid chromatography (HPLC) to the determination of vitamins in food, it has been rightly stated that the main problem is not the separation of vitamins from each other but from the complex matrices[1]. To solve such a problem, sample purification prior to analysis was carried out using column chromatography[2] or HPLC[3], but such purification steps always require manipulation and are time consuming; whenever possible they should therefore be avoided in order to enhance the advantages of speed and simplicity of the HPLC technique.

Some papers have dealt with the determination of more than one vitamin (*e.g.*, A and E[4], A and D_3[5], A,E and D_3[6], A, D_2 or D_3, E and K_1[7]) in various matrices by HPLC.

The complete analysis of all fat-soluble vitamins in a food sample is also complicated by the fact that vitamin A is generally present in much larger amounts than D vitamins. Therefore, in a previous paper the analysis of vitamin A together with its geometric isomers and carotene (which were quantified in cheese samples) was considered[8]; the successive separation of fat-soluble vitamins (A, D_2, D_3, E) in oils without quantification was described[9].

This work was aimed at the simultaneous quantification of vitamins D_2, D_3 and E in cod liver oil sample. Although vitamin D_2 comes from vegetable sources and D_3 from animal sources[10], so that they are unlikely to occur together in a food sample, we analysed both D vitamins because our chromatographic system was able to resolve this pair of substances well. Therefore, vitamin D_2 was added and re-covered from cod liver oil which naturally contains only vitamin D_3[3]. The use of the same matrix for testing percentage recoveries of the analytes is useful in comparing the relative behaviours of the two D vitamins.

The method adopted is simple as purification steps have been avoided, and the extraction procedure was studied in order to obtain better recoveries of the analytes.

EXPERIMENTAL

Apparatus

The liquid chromatographic apparatus was as described previously[11]. Two reversed-phase columns connected in series were used: (1) Perkin-Elmer (Norwalk, CT, U.S.A.) ODS-HC Sil-X-1 (25 × 0.26 cm I.D.) and (2) Supelco (Bellefonte, PA, U.S.A.) Supelcosil LC18 (15 × 0.46 cm I.D.).

A Supelco guard column dry-packed with 40-μm LC18 pellicular packing was also employed.

Peak areas were computed by means of a Sigma 15 chromatography data station (Perkin-Elmer).

Reagents and materials

Vitamin D_2 (E. Merck, Darmstadt, G.F.R.), vitamin D_3 (E. Merck) and DL-α-tocopherol (Sigma, St. Louis, MO, U.S.A.) were used for preparing stock standard solutions in HPLC-grade methanol (E. Merck). The concentrations of the above solutions were 100 mg per 100 ml for vitamins D_2 and D_3 and 250 mg per 100 ml for vitamin E.

A dilute working solution of all three vitamins was prepared by adding 10 ml of vitamin D_2, 10 ml of vitamin D_3 and 50 ml of vitamin E stock-standard solutions to a 100-ml volumetric flask, which was then made up to the mark with methanol. The resulting concentration of the working solution was therefore 10 mg per 100 ml for vitamins D_2 and D_3 and 125 mg per 100 ml for vitamin E. This working solution was used to obtain calibration graphs and for the standard additions technique.

Methanol, acetonitrile and methyl ethyl ketone were of HPLC grade (E. Merck). Hexane of extra-pure grade (E. Merck) and diethyl ether of ACS grade (Riedel-de Haën, Hannover, G.F.R.) were used for extractions without any further purification.

L-(+)-Ascorbic acid (extra pure; E. Merck), potassium hydroxide pellets (GR; E. Merck), anhydrous sodium sulphate (RPE; Carlo Erba, Milan, Italy), monobasic sodium phosphate and dibasic sodium phosphate (RP; Carlo Erba), and absolute ethanol (RPE; Carlo Erba) were also employed.

Sample preparation

Alkaline digestion. A 10-g amount of oil was used for alkaline digestion. The sample was weighed into a 250-ml erlenmeyer flask and 0.5 g of ascorbic acid (as

antioxidant[9,12]), 25 ml of distilled water, 50 ml of ethanol and 25 ml of potassium hydroxide solution (100 g of potassium hydroxide in 100 g of water) were added. A magnetic stirrer was inserted and the flask was flushed with nitrogen and stoppered. Alkaline digestion was carried out with magnetic stirring at room temperature overnight[9].

Extraction procedure: method A —with hexane. The digested alkaline solution was poured into a 500-ml separating funnel and extracted four times (2 min shaking) with four 50-ml portions of hexane. The extracts, pooled into another separating funnel, were washed three times with 50-ml portions of dilute phosphate buffer (pH 7.4).

The washed extracts were then filtered through a Schleicher & Schüll No. 589 filter-paper containing 30 g of anhydrous sodium sulphate into a 250-ml round-bottomed flask, and the filter was rinsed twice with 10-ml portions of hexane. The solution was evaporated by means of a rotary vacuum evaporator (water-bath temperature $\leqslant 40°C$) and the residue was transferred by quantitatively rinsing the flask with methyl ethyl ketone into a 5-ml volumetric flask. A 50-μl volume of this solution was used for chromatographic injections.

Method B —with diethyl ether. The digested alkaline solution was poured into a 500-ml separating funnel and extracted once with 200 ml of diethyl ether. The two phases were allowed to separate and the aqueous layer was discarded. The organic layer was then washed with phosphate buffer and the procedure was carried out as described above (rinsing the filter with diethyl ether) for method A.

Chromatographic conditions

The acetonitrile–methanol–water eluent, the gradient of elution, the wavelength changes and other chromatographic parameters were described previously[9].

Data processing

The linearity of the calibration graphs and of the graphs obtained by means of the standard additions technique was tested by analysis of the variance (ANOVA) for the linear regression, using F and R^2 ratios as criteria of adequacy, where F is the ratio between the variance attributable to regression and the variance attributable to deviation from regression, and R^2 is the ratio between the sum of squares attributable to regression and the total sum of squares[13]. Computations were performed on an Olivetti P6040 desk calculator.

RESULTS AND DISCUSSION

An extraction method (method B) that is very rapid and reliable owing to the very few manipulations required was adopted. The higher polarity of diethyl ether compared with hexane resulted in higher recoveries of analytes with only one extraction instead of four. Also, the use of the buffer instead of water for washings and redissolution of the samples in methyl ethyl ketone instead of methanol were effective in avoiding problems that had sometimes previously been encountered (such as soap hydrolysis and turbidity of extracts during washing and precipitation of samples during redissolution). Of course, the simplicity of the method could also be regarded as limiting when trace analysis is required; in this instance concentration steps could be required, which were not taken into consideration in this study.
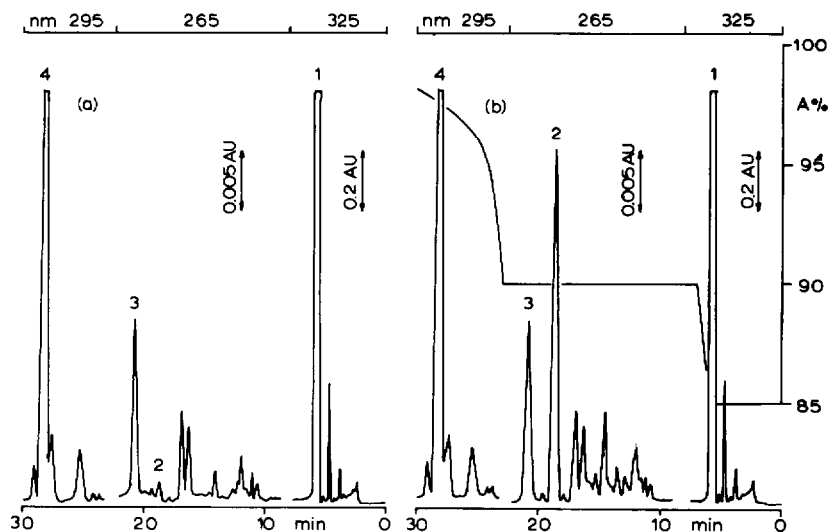
Fig. 1. (a) Chromatogram of an extract of cod liver oil, injected immediately after its preparation. (b) Chromatogram of the same sample, injected about 48 h later. Peaks: 1 = vitamin A; 2 = x-tocopherol-quinone (formerly U1[9]); 3 = vitamin D_3; 4 = vitamin E. Gradient elution programme: percentage of mixture A (methanol–acetonitrile, 20:80) referred to the total volume of A + B (B is water) is reported superimposed. Wavelength changes are reported at the top of the figure. Flow-rate, 1.5 ml/min; the pressure drop ranged from 9.8 to 6.5 MPa according to the gradient programme. Temperature: 44°C. Sensitivity of the recorder: 2 and 0.05 a.u.f.s. Chart speed: 5 mm/min. Injection: 50 μl.

Chromatographic profile and peak identification

The chromatographic profile obtained with injection of cod liver oil extract, peak identification and effects of the chromatographic conditions have been considered in a previous paper[9]. The same conditions were employed for this study on vitamin quantitation.

The order of elution of vitamins D_2, D_3 and E does not follow their relative polarities. Owing to the double bond in the C_{22}–C_{23} position, vitamin D_2 has a slightly higher polarity than D_3, and is eluted sooner. Vitamin E, which is more polar than the two D vitamins (dissolved and extracted much better in diethyl ether than hexane, as shown by recovery data) is eluted much later. The main factor influencing the elution order in this instance is the long hydrocarbon chain of vitamin E, which is strongly retained by the C_{18} stationary phase. A rationalization of the chromatographic behaviour of vitamin D_2 and D_3 and related compounds on silica phases, in terms of relative molecular planarity, has been published[14]. With reversed phases, with long bonded hydrocarbon chains, the concept of planarity can be integrated with that of chain length.

Together with the peaks of the vitamins, a large unknown peak (U1) had previously been reported and the shape of its spectrum in the region between 230 and 300 nm had been discussed[9]. It has now been found that this peak was formed in the 5-ml flask containing the sample, on standing, so that whereas it was hardly detectable in the first injection, its area increased in subsequent injections (every injection takes about 1 h, because after elution of the sample a purge and an equilibration programme of 15 min each are needed). Fig. 1 shows two chromatograms of the same
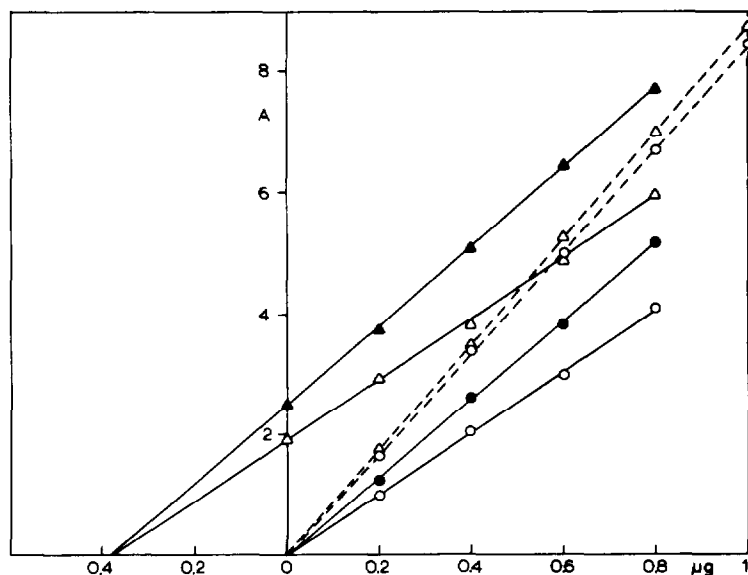
Fig. 2. Calibration graphs (broken lines) and graphs obtained by means of the standard additions technique for vitamins D_2 (○) and D_3 (△) in cod liver oil. Open symbols refer to method A (with hexane) and closed symbols to method B (with diethyl ether). Micrograms (abscissa) are plotted *versus* area counts (ordinate).

sample of cod liver oil extract; the first (a) was obtained on injecting the sample immediately after its preparation and the second (b) after standing. Therefore, this peak has been assigned to an oxidation product which is formed after extraction. We have collected sufficient evidence to identify such a peak with the oxidized form of α-tocopherolquinone. Further studies on such oxidation products are in progress. However, as the vitamin peak areas did not change, such oxidation products do not appear to interfere with the analytes considered here and the quantitative results were not affected.

Calibration graphs and standard additions technique

The dilute vitamin standard solution (see *Reagents and materials*) was used to obtain calibration graphs (injecting different volumes from 2 to 20 μl) and for the standard additions technique. In the latter instance aliquots from 200 to 800 μl of the solution were added to the samples prior to alkaline digestion.

Calibration and standard addition graphs are reported in Figs. 2 and 3, which refer to D vitamins and to vitamin E, respectively.

Table I shows the values of the parameters a and b of the equation $y = a + bx$, where y is the peak area and x is the amount of analyte injected in micrograms. The values of the standard deviation of the slope, s_b, and of the intercept, s_a, and F and R^2 test values are also reported. Values of z represent the actual amount of vitamins in a sample, and are given by the fraction $|-a/b|$.

The above parameters were obtained by applying both extraction methods (A and B) and refer to standard additions technique graphs for the three vitamins and to

TABLE I

VALUES OF a, b, s_a, s_b AND z, OF F AND R^2 TESTS AND OF PERCENTAGE RECOVERIES ($Q \pm s_Q$)

The values refer to the calibration graphs for vitamins D_2, D_3 and E and to the standard additions technique graphs for the analytes in cod liver oil samples. As $y = a + bx$ is the general equation of the graphs, s_a and s_b represent the standard deviation of the intercept and of the slope, respectively. z is the value of x when $y = 0$ and is given by the ratio $\left| -\dfrac{a}{b} \right|$, s_z being its standard deviation. s_Q is the standard deviation of the percentage recovery of analyte from the sample considered. Recovery values are obtained dividing b values of sample by the corresponding b value of the calibration graph.

| Procedure	Analyte	a	s_a	b	s_b	$z \pm s_z$	F	R^2	$Q \pm s_Q$
Calibration	Vitamin D_2	0.023	0.046	8.455	0.070		14515	0.9997	
	Vitamin D_3	0.017	0.038	8.785	0.058		23210	0.9998	
	Vitamin E	0.033	0.126	1.221	0.009		19076	0.9997	
Extraction, method A (with hexane)	Vitamin D_2	−0.013	0.033	5.120	0.067		5868	0.9995	60.6 ± 1.0
	Vitamin D_3	1.882	0.050	5.000	0.103	0.376 ± 0.013	2366	0.9987	56.9 ± 1.2
	Vitamin E	2.834	0.049	0.172	0.008	16.477 ± 0.811	465	0.9936	14.1 ± 0.7
Extraction method B (with diethyl ether)	Vitamin D_2	−0.014	0.038	6.520	0.079		6747	0.9996	77.1 ± 1.1
	Vitamin D_3	2.476	0.029	6.570	0.060	0.377 ± 0.006	11880	0.9997	74.8 ± 0.8
	Vitamin E	18.960	0.096	1.096	0.016	17.299 ± 0.264	4871	0.9994	89.8 ± 1.4
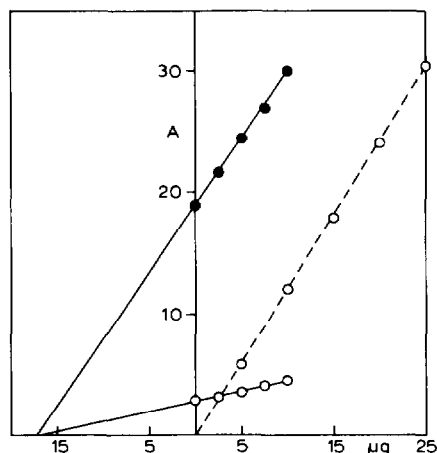
Fig. 3. Calibration graph (broken line) and graphs obtained by means of the standard additions technique for vitamin E in cod liver oil. Symbols and axes as in Fig. 2.

their calibration graphs. Linearity of the graphs in the concentration range used shows that the recovery of the analyte is unaffected by their concentrations.

The percentage recoveries (Q) are strongly dependent on the extraction solvent, with the greatest difference being attributable to vitamin E: only 14% was recovered with hexane whereas almost 90% was recovered with a single diethyl ether extraction (method B). This can be clearly seen in Fig. 3 from the great difference in the slopes of the two lines obtained with hexane and diethyl ether as extracting solvent; both lines have almost the same intercept on the abscissa (see Table I), which represents the amount in micrograms of the vitamin contained in the injected sample. To obtain concentration values, expressed in micrograms per 100 g of sample, the values of z obtained are multiplied by 1000, because the extract from 10 g of sample was dissolved in 5 ml and an aliquot of 50 μl of this volume was injected.

Table II reports the vitamin concentrations found in the cod liver oil sample, compared with some concentrations and concentration ranges reported in the literature[3,15]. The intra-laboratory repeatability of the proposed method, expressed as the coefficient of variation, is about 1.7 and 1.6% for vitamin E and vitamin D_3, respectively.

TABLE II

VITAMIN E AND VITAMIN D_3 CONCENTRATIONS IN COD LIVER OIL

Values found applying the proposed method are reported and compared with literature data[3,15].

Vitamin	Found	Literature value
Vitamin E (mg per 100 g)	17.3 ± 0.3	21.4 (9.7–32.2)[15]
Vitamin D_3 (μg per 100 g)	377 ± 6	491 (258–800)[3]

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