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QUANTITATIVE ANALYSIS OF PHYLLOQUINONE (VITAMIN K_1) IN SOY BEAN OILS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for determining phylloquinone (vitamin K_1) in soy bean oils is described. Resolution of vitamin K_1 from interfering peaks of the matrix was obtained after enzymatic digestion, extraction and liquid–solid chromatography on alumina. An isocratic reversed-phase chromatography with UV detection was used in the final stage. The quantitation was carried out by the standard addition method, and the recovery of the whole procedure was 88.2%.

INTRODUCTION

The literature up till 1980 on the high-performance liquid chromatography (HPLC) of vitamins K, has been reviewed by Shearer[1]. Several papers[2–6] contributed to knowledge about the chromatographic behaviour of phylloquinone (vitamin K_1) and related substances, but the HPLC separations mainly concerned mixtures of pure standards. There have been few analyses of biological matrices[4,6], and those of foods have been restricted to some common vegetables, milk and infant food formulae[7–11].

We have developed an HPLC method for determining the vitamin K_1 content of soy bean oils, which are among the richest nutritional sources of vitamin K[12]. Suitable samples for HPLC were obtained by enzymatic digestion of common commercial oils, followed by extraction and a further purification by liquid–solid chromatography (LSC) on alumina. They were then chromatographed on a reversed-phase C_{18} column eluted with an isocratic ternary mobile phase which permitted a complete resolution of phylloquinone from other peaks of the matrix.

EXPERIMENTAL

Apparatus

The chromatographic apparatus consisted of a pump module (Series 10 liquid chromatograph; Perkin-Elmer, Norwalk, CT, U.S.A.), a variable-wavelength spectrophotometer (LC 75, Perkin-Elmer) and a data processor (Chromatopac C-R1B; Shimadzu, Kyoto, Japan).

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For recording UV–visible spectra of the eluted peaks, a spectrophotometer (LC 55 B, Perkin-Elmer) connected to a digital scanner (LC 55S, Perkin-Elmer) and a recorder (Model 56, Perkin-Elmer) was employed.

A guard column (5 × 0.4 cm I.D.) (Supelco, Bellefonte, PA, U.S.A.) dry packed with 40-μm C_{18} pellicular packing was connected to the analytical column (15 × 0.46 cm I.D.) containing 5-μm porous C_{18} particles (Supelco).

A UV–visible spectrophotometer (Super Scan Model 3; Varian Techtron, Springvale, Australia) was also employed.

Reagents

A standard of vitamin K_1 (purum) was purchased from Fluka (Buchs, Switzerland). The lipase (E.C. 3.1.1.3) (Type VII from *Candida cylindracea*) was obtained from Sigma (St. Louis, MO, U.S.A.).

Ethanol, methanol and acetonitrile (HPLC grade), aluminium oxide (standardized for adsorption chromatographic analysis according to Brockmann, activity II–III) and potassium hydroxide (pellets, reagent grade) were obtained from E. Merck (Darmstadt, F.R.G.). *n*-Hexane (HPLC grade), *n*-pentane and diethyl ether (RPE), anhydrous sodium sulphate, disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Carlo Erba (Milan, Italy).

Oil samples

Commercial soy bean oil samples were purchased at random in grocery stores: 1, Crivellaro S.p.A.-Padova, expiry date November 1985; 2, "Friggi", Italiana Olii e Risi S.p.A.-Ravenna, expiry date January 1986; 3, Erbifori & Viola-Bardolino, Verona, expiry date 1986; 4, Venturi, Spoleto, expiry date March 1986.

Methods

A standard solution of vitamin K_1 (10 mg per 100 ml) in *n*-hexane was prepared in an amber coloured volumetric flask. Its concentration was estimated by measuring the absorbance [after suitable dilution (1:8, *i.e.*, 1.25 mg per 100 ml) using 1-cm cells] which was found consistent (0.526) with that calculated (0.525) appling the extinction coefficient, $E^{1\%}_{1\,cm,\ \lambda 249\ nm} = 420$, reported previously[13]. The standard solution, kept at 4°C, was periodically examined by HPLC and found to be stable for over a month.

Sample analyses were performed according to the standard addition method, pipetting aliquots (0.5, 1.0 and 1.5 ml) of the vitamin K_1 standard solution into three 250-ml amber flasks. The hexane was evaporated with a nitrogen stream before weighing into each flask the same quantity (15 g) of oil. A blank oil sample, without added vitamin K_1, was simultaneously subjected to the same analytical procedure.

A 7.5-g amount of lipase and 25 ml of phosphate buffer (pH 5.5) were added to each sample and the enzymatic digestion allowed to proceed with magnetic stirring overnight (about 15 h at 37°C). After cooling, the mixtures were transferred (washing with 25 ml of phosphate buffer, pH 5.5) into ambered 250-ml separating funnels, and extracted with 100 ml of *n*-pentane. Before separating the organic from the aqueous layers, 50 ml of ethanolic potassium hydroxide solution [7 ml (100 g per 100 ml) of an aqueous solution plus 43 ml of ethanol] were added to each separating funnel with shaking in order to obtain the alkaline salts of the hydrolysed fatty acids. The

pH of the aqueous layer was then strongly basic and the separation of the layers had to be performed with minimum delay as vitamin K_1 is rapidly destroyed in alkaline media. The aqueous layers were re-extracted with 50 ml of *n*-pentane and the pooled organic extracts washed twice with 100-ml aliquots of phosphate buffer (pH 7.7, in order to avoid soap hydrolysis) and once with 100 ml of distilled water. The organic extracts were dried by adding to each sample 20 g of anhydrous sodium sulphate, filtered (on paper, 589 Schwarzband; Schleicher & Schüll, Dassel, F.R.G.) into amber flasks and then evaporated using a rotary vacuum evaporator.

The samples must be dry before performing LSC: a simple test is that the extracts, when redissolved with hexane in a 5-ml volumetric flask, are completely limpid, with no trace of turbidity. These solutions were loaded on an alumina column (14 × 1.5 cm I.D.) and eluted first with diethyl ether in hexane (7%, v/v), discarding the first 45 ml. The next 50 ml were collected, evaporated and redissolved with hexane in a 5-ml volumetric flask. A 25-μl aliquot was used for HPLC.

Data processing

Statistical Analysis of the data was performed with a Olivetti M 20 personal computer. The linearity of the calibration graph and of the standard addition graphs was tested by analysis of ther variance (ANOVA) for the regression, using R^2 and F ratios as criteria of adequacy[14]; R^2 is the ratio of the sum of squares attributable to regression and the total sum of squares, and F is the ratio of the variance attributable to regression and the variance attributable to the deviation from regression.

RESULTS AND DISCUSSION

The main problem in the chromatographic separation of vitamin K_1 from natural matrices is the difficulty of removing the bulk lipids of the samples, which strongly interfere by coeluting and frustrating the HPLC separation. While alkaline digestion is a very effective method of sample purification in the case of the three other fat-soluble vitamins (A, D and E), vitamin K is rapidly destroyed by alkali and alternative methods must be employed. Barnett *et al.*[9] were probably the first to propose and apply enzymatic digestion of the sample for analyzing infant food formulae, followed by Bueno and Villalobos[11]. We adopted a slightly modified enzymatic digestion (pH 5.5 instead of 7.7) for soy bean oil samples, thus obtaining a preliminary purification.

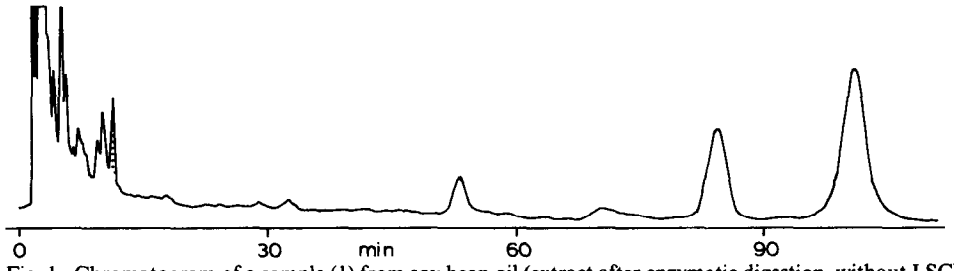

Fig. 1. Chromatogram of a sample (1) from soy bean oil (extract after enzymatic digestion, without LSC). The marked peak corresponds to the partially resolved vitamin K_1. Isocratic mobile phase: methanol–acetonitrile–water (88:10:2), flow-rate 1.5 ml/min. Detection wavelength: 270 nm. Amount injected: 25 μl.

Fig. 1 shows a chromatogram obtained for an extract of the enzymatically digested sample. This result is unsatisfactory for two reasons. (a) The marked peak, with a retention time corresponding to that of vitamin K_1, is incompletely resolved. It was also shown to be a composite one, with a coeluting contaminant, when absorbance spectra were recorded by means of the flow-stop method. Chromatographic resolution could not be improved by changing the mobile phase composition. (b) There are late eluting peaks (with retention times longer than 100 min) which would require a gradient elution followed by an equilibration step before repeating the injection. This would be very time-consuming.

Both problems were circumvented by LSC. Alumina was chosen as the column packing owing to its ease of packing with respect to silica and because it also permits greater gravity flow-rates. The appropriate fraction of the eluate (containing vitamin K_1) was determined by examination of the behaviour of the standard solution by HPLC. Note that the volume collected may vary from one batch of alumina to another, and that it should be sufficient to guarantee a complete recovery of vitamin K, while minimizing the amount of interfering substances. A suitable compromise was achieved by adopting the conditions described in the Experimental section.

Hexane had to be used as the final solvent in order to permit a ready and complete dissolution of the purified sample. Its use in the reversed-phase system (although hardly satisfactory) resulted in only slightly broader vitamin K peaks as compared to injections of the standard in methanol solution.

Having at hand an hexane solution of the sample ready for HPLC, we were prompted to test also a silica column (Si 60, Merck) with hexane–*n*-octanol (99.8:0.2) as mobile phase, flow-rate 1.5 ml/min. Under these conditions, vitamin K_1 was again coeluted with interfering peaks of the matrix, and the use of a reversed-phase system was thus unavoidable.

Fig. 2 shows the reversed-phase chromatogram obtained for the sample after

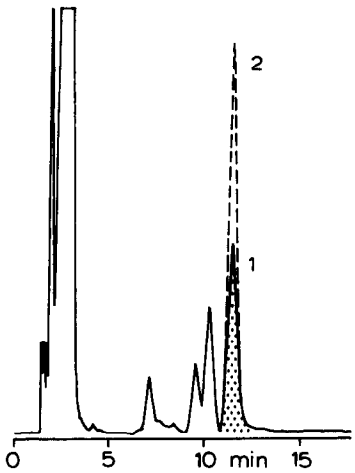

Fig. 2. Chromatogram of a sample (1) from soy bean oil (extract after enzymatic digestion and purification by LSC on alumina). Peaks: 1 = vitamin K_1 endogenous to the oil; 2 = endogenous vitamin K_1 plus vitamin K_1 added (50 μg to the sample, *i.e.*, 0.25 μg in 25 μl) for determining recovery. Chromatographic conditions as in Fig. 1.

the LSC purification. The vitamin K_1 peak is now completely resolved from previous coeluting substances and its purity was demonstrated by recording its absorbance spectrum. This resolution is obtainable by using a ternary isocratic mobile phase (methanol–acetonitrile–water, 88:10:2). Purge and equilibration steps are not required as the formerly late-eluting peaks (see Fig. 1) were swept away by the LSC. Repeated injections of samples are therefore possible, and a chromatographic analysis is completed isocratically in less than 15 min.

Quantitative analysis

The concentration of phylloquinone was determined by applying the standard addition method. Fig. 3 shows the graph (●) obtained by plotting the vitamin K_1 peak area values *versus* the known added quantities (μg, in 25 μl injected) of the standard. A calibration graph (○) was also determined by injecting the same standard solution used for the additions.

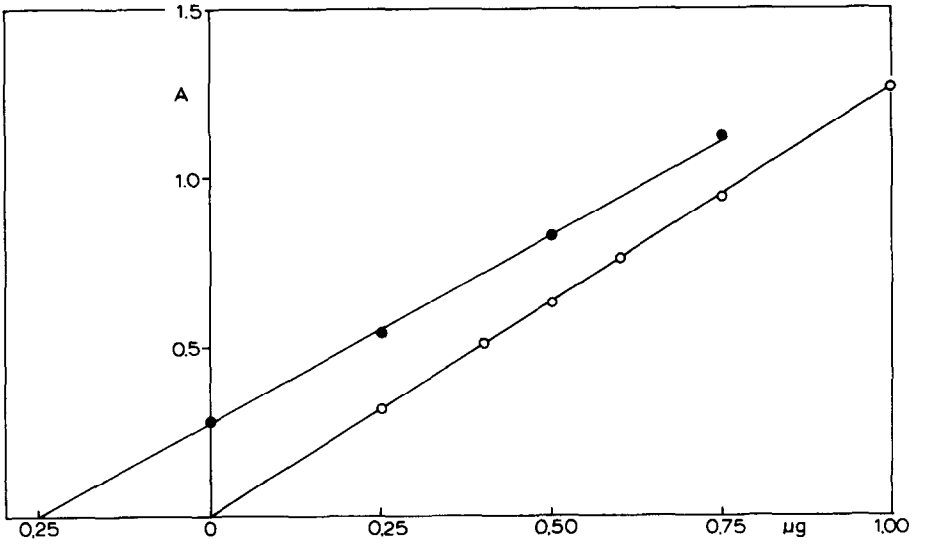

Fig. 3. Calibration graph (area count *vs.* analyte amount) for phylloquinone (○) and graph obtained by means of the standard addition method (●) applied to oil sample 1. The area count values, multiplied by 10^5, correspond to the output of the integrator used.

From the ratio of the slope of the standard addition graph to that of the calibration graph, the recovery of the whole analytical procedure (enzymatic digestion, extraction, LSC purification) was obtained. Its value was 88.2% as a mean for four analyses of different samples.

Table I shows all the parameters of the linear regression of the graphs, as well as the concentrations of vitamin K_1 in the samples. These concentrations vary over a rather large range from 121 to 333 μg per 100 g, probably as a consequence of the industrial methods used for producing the oils and of the quality of the raw materials used. The concentrations found by us are also lower than those previously reported for soy bean oils[12]. We do not know yet whether this is attributable to a difference intrinsic to the different oil samples or to the different analytical methods.

TABLE I

PARAMETERS OF THE CALIBRATION GRAPH AND OF THE STANDARD ADDITION GRAPHS FOR VITAMIN K_1

The values for the standard addition graphs refer to the four different soy bean oils. The general equation of the graphs is $y = a + bx$, where a and b represent the intercept and the slope respectively; q, defined as the absolute value of the ratio $-a/b$, represents the amount (μg) of the analyte contained in the volume (25 μl) of the sample injected and Q is the resulting concentration of vitamin K_1 in the oil (μg/100 g). Recoveries, P, were obtained dividing the b values for the standard addition graphs by that corresponding to calibration graph. s is the standard deviation.

Graph	*Compound*	$10^5\ a$	$10^5\ s_a$	$10^5\ b$	$10^5\ s_b$	F	R^2	$q \pm s_q$	$Q \pm s_Q$	$P \pm s_P$
Calibration	Vitamin K_1	0.000	0.006	1.270	0.010	16 429	0.9998			
Standard additions	Sample 1	0.279	0.007	1.114	0.015	5236	0.9996	0.250 ± 0.007	333 ± 9	87.7 ± 1.4
	Sample 2	0.217	0.007	1.125	0.016	5122	0.9995	0.193 ± 0.007	257 ± 9	88.6 ± 1.5
	Sample 3	0.103	0.010	1.126	0.020	3077	0.9993	0.091 ± 0.009	121 ± 12	88.7 ± 1.7
	Sample 4	0.170	0.008	1.117	0.018	3818	0.9994	0.152 ± 0.008	203 ± 11	87.9 ± 1.6

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