SYMPOSIUM: *IN VIVO* ANTIOXIDANTS AND POLYUNSATURATED ACID METABOLISM

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The Application of Gas-Liquid Chromatography to the Determination of Vitamins E and K[1]

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Abstract

The separation and quantitation of vitamins E and K have been made possible by gas-liquid chromatography, using a mixture of two different silicone rubber polymers as the liquid stationary phase. α-Tocopherolquinone and α-Tocopherolhydroquinone could be separated from the original α-tocopherol by this technique. Crude lipid extracts from mammalian tissues contain an unknown component which converts α-tocopherolquinone to α-tocopherol during chromatography. The implications of this observation is discussed in the light of biological processes. A group of derivatives, with the trimethylsilyl ether link was prepared and characterized by gas chromatography and infrared spectrophotometry.

Introduction

THE ROLE of vitamins E and K in biological processes have been fairly extensively studied from various standpoints. However, most of the reported work is based on methodology fraught with many pitfalls. There are three main problems encountered in the determination of these vitamins. The first of these is the extraction of the vitamin from the biological material, quantitatively. Second, involves the precautions to be taken during processing, such as saponification and crystallization. Third, and most important, is the actual quantitation of the vitamin by a sensitive and specific method. Several techniques employed recently involved chemical reactions (1–3), paper chromatography (4), fluorescence spectroscopy (5), and thin layer chromatography (6). The first report of the use of gas-liquid chromatography was indicated by Nicolaides (7). The present paper describes a sensitive technique for the determination of the individual tocopherols and vitamin K, using gas-liquid chromatography. In addition to being highly sensitive, the compounds could be recovered and subjected to confirmatory chemical tests.

Materials and Methods

Extraction: The extraction procedure of Edwin et al. (4) was found to be satisfactory.

Silicic acid chromatography: Dry silicic acid of chromatographic grade (Unicil) was suspended in isooctane and packed into a column 6 × 1 cm, and 10–12 mg of either saponified or unsaponified lipid was applied on the column and washed with 100 ml of isooctane. The tocopherols were then eluted with 150 ml of 10% ethyl acetate in isooctane and concentrated in a rotary evaporator at low temperature. Flow rate was of the order of 2 ml/min.

Precipitation of sterols: Interfering sterols when present after silicic acid chromatography were precipitated out from a 5% methanolic solution at −10C.

Gas chromatography: Gas Chrom P, 60–80 mesh (Applied Science Laboratories) was suspended in concentrated hydrochloric acid overnight. The acid was decanted off and the support washed six times with hydrochloric acid. The last acid wash should have no yellowish tinge. The support was then repeatedly washed with distilled water, until neutral,

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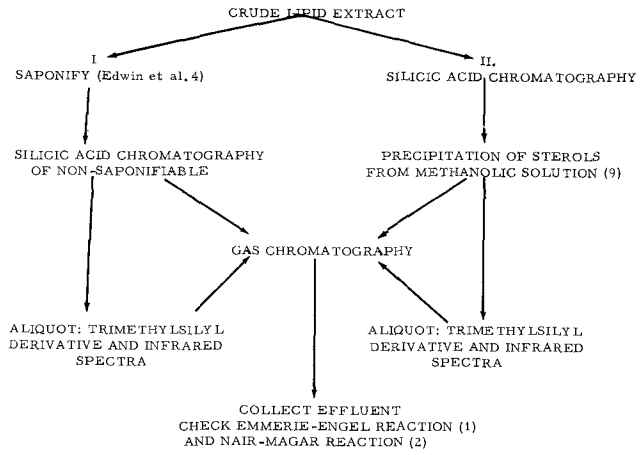


FIG. 1. Flow sheet showing alternate procedures applicable to biological material prior to gas chromatography.

washed with acetone and then dried in an oven at 90C.

50 g of the dried support was then treated with 200 ml of a 5% solution (v/v) of dimethyl-dichlorosilane (General Electric Co.) in toluene under intermittent water pump vacuum for 10 min, filtered through a Buchner funnel and washed with an excess of analytical grade methanol. The siliconized support was dried at 90C for about 4 hr with gentle tapping at short intervals.

Two special types of silicone rubbers were used as the stationary phase. One of them designated SE 52, was a methyl silicone rubber gum consisting of 5 mole % phenyl groups and the other, designated 287-108-949 was a nitrile polysiloxane rubber consisting of 50 mole % methyl β-cyanoethyl methyl siloxane and 50 mole % dimethyl β-siloxane. 2.5 g of SE 52 and 2.5 g of 287-108-949 were dissolved in 200 ml of toluene and used to treat 25 g of siliconized support, under intermittent partial vacuum. The coated support was dried in an oven at 90C for about 4 hr, and packed in a 183 cm × 5 mm column. A Barber-Colman Model 10 apparatus with an argon ionization detector, (strontium foil) was used. The column was initially conditioned for about 3 days at 240C. There was practically no bleeding of the phase, and was taken to signify the success of the procedure.

Trimethyl silyl (TMS) ethers of tocopherols: The sample containing about 5 mg of standard tocopherol in about 5 ml of chloroform was treated overnight with 0.2 ml of hexamethyldisilazane (General Electric) and five drops of trimethylchlorosilane (General Electric) as the catalyst. The reaction mixture was evaporated to dryness under a stream of N_2 and extracted with a small volume of benzene. The quantitative formation of the derivative was confirmed by infrared spectra with a Beckman Model IR_4 spectrophotometer. The spectra were obtained using the potassium bromide micropellet technique at a concentration of 1.25–1.5%, and a light path of approximately 0.16 mm.

TABLE I

Retention Time of Components in a Standard Mixture

No.	Compound	Tr [a]	TR [b]
		Min	
1	Pentamethyl 6-hydroxychroman	1.5	0.05
2	Squalene	8.8	0.28
3	Metabolite of Simon (8)	13.4	0.49
4	Δ-Tocopherol	26.0	0.81
5	β-Tocopherol	29.4	0.92
6	γ-Tocopherol	29.4	0.92
7	α-Tocopherol	32.0	1.00
8	α-Tocopherolquinone	39.6	1.24
9	α-Tocopherolhydroquinone	39.6	1.24
10	Vitamin K_1	53.2	1.66

[a] Tr: Retention time in minutes.
[b] TR: Retention time relative to α-Tocopherol.
Column temperature 228C; Flow rate 75 ml/min. Gain 3×10^{-8} AMP.

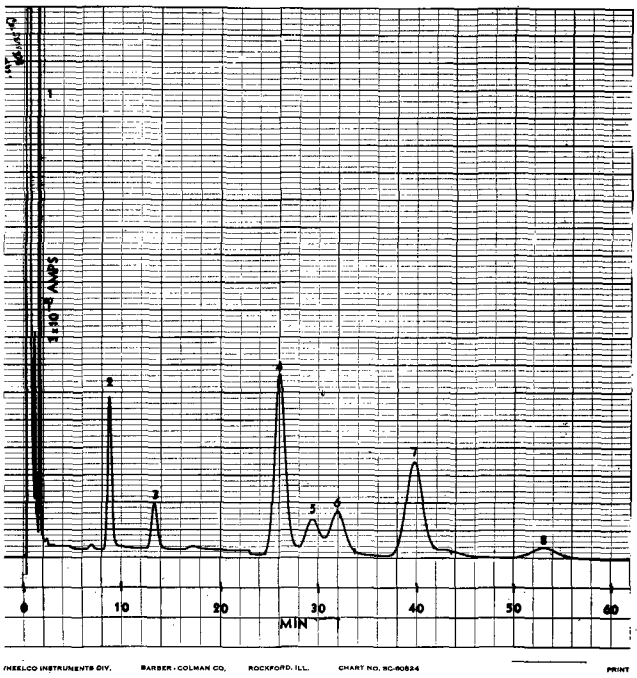


FIG. 2. Gas chromatography of a standard mixture. Column temperature 228C, cell temperature 250C, Argon flow rate 75 ml/min, Gain 3×10^{-8} AMP. 1. Pentamethyl hydroxychroman 2. Squalene; 3. Simon's metabolite (8); 4. Δ-Tocopherol; 5. β + γ Tocopherol; 6. α-Tocopherol; 7. α-Tocopherolquinone + α-Tocopherolhydroquinone; 8. Vitamin K_1.

Results and Discussion

Figure 1 is a flow diagram indicating alternative procedures for extraction and purification of biological material prior to gas chromatography. Initial extraction has to be carried out under very mild conditions with adequate precautions to prevent oxidation of vitamins E and K. The extracted lipid is kept in an atmosphere of nitrogen and covered with black foil to prevent photochemical degradation.

Under our standard conditions of chromatography at a column temperature of 228C and flow rate of 75 ml/min, the values for retention time are as in Table I. Figure 2 is a representative chromatogram showing the separation of components in a standard mixture. The vitamin E nucleus, 2,2,5,7,8-pentamethyl-6-hydroxychroman is the first component to

TABLE II

Gas Chromatography of the Trimethylsilyl Ethers of Tocopherols

No.	Compound	Tr [a]	TR [b]
		Min	
1	TMS of Pentamethyl 6-hydroxychroman	1.1	0.04
2	Pentamethyl 6-hydroxychroman	1.4	0.05
3	Squalene	9.1	0.32
4	TMS-Δ-Tocopherol	12.4	0.44
5	TMS-β-Tocopherol	15.7	0.55
6	TMS-γ-Tocopherol	15.7	0.55
7	Δ-Tocopherol	21.5	0.76
8	TMS-α-Tocopherol	22.9	0.81
9	β-Tocopherol	25.1	0.89
10	γ-Tocopherol	25.1	0.89
11	α-Tocopherol	28.3	1.00
12	α-Tocopherolquinone	33.7	1.19
13	α-Tocopherolhydroquinone	33.7	1.19
14	Vitamin K_1	45.2	1.59

[a] Tr: Retention time in minutes.
[b] TR: Retention time relative to α-Tocopherol.
Column temperature: 237C; Cell temperature 250C; Flow rate 75 ml/min; Gain 1×10^{-8} AMP.

come off the column, followed by squalene which is used as a marker in this standard mixture to monitor performance throughout the life of the column. A metabolite of vitamin E isolated by Simon et al. (8), Δ-tocopherol, β + γ tocopherol, α-tocopherol, α-tocopherolquinone + α-tocopherolhydroquinone and vitamin K_1, appear in the indicated sequence.

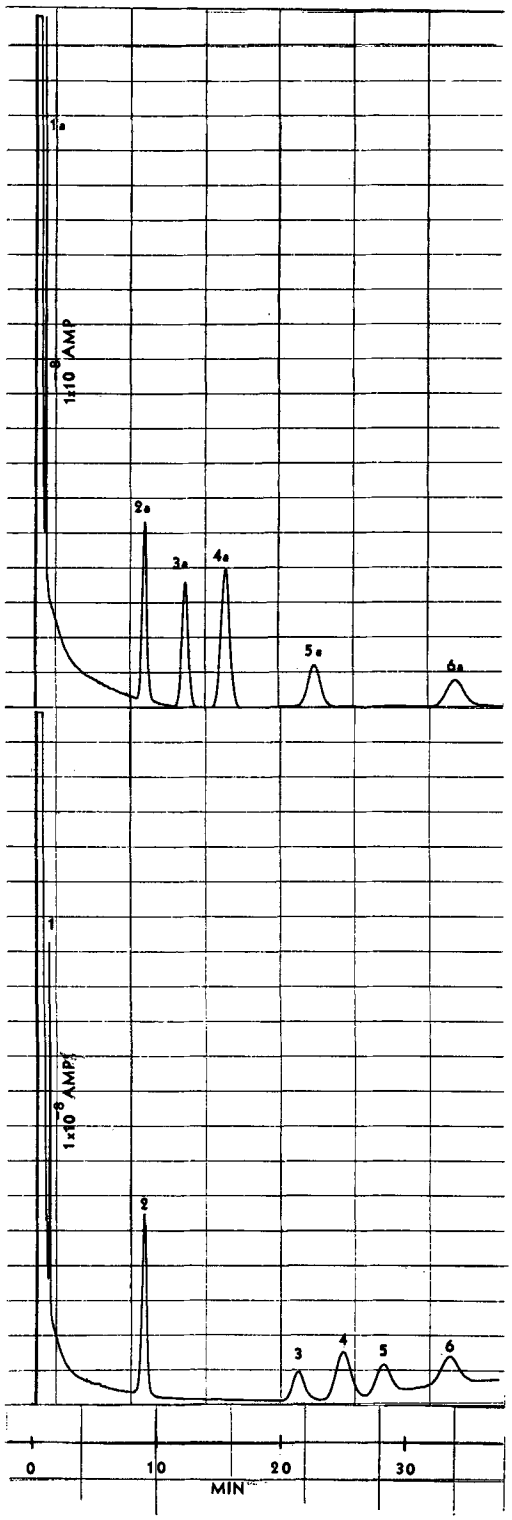


FIG. 3. Gas chromatography of the tocopherols and their trimethylsilyl ethers. 1A: Pentamethyl hydroxychroman (silyl ether); 2 and 2A: Squalene; 3A: Δ-Tocopherol (silyl ether); 4A: β + γ-Tocopherol (silyl ethers); 5A: α-Tocopherol (silyl ether); 6 and 6A: α-Tocopherolquinone and α-Tocopherolhydroquinone. 1–6: Same mixture as 1A–6A before preparation of silyl ethers. Column temperature 237C; cell temperature 250C, Argon flow rate 75 ml/min; Gain 1×10^{-8} AMP.

TABLE III

Effect of Crude Beef Heart Lipid when Mixed with Authentic α-Tocopherolquinone

Substance	Area under curves in units	
	α-Tocopherol	α-Tocopherol-quinone
1. Crude beef heart lipid-2λ[a]	16.83	.....
2. Standard α-Tocopherolquinone	.....	10.12
3. Crude beef heart lipid 2λ plus standard α-Tocopherolquinone 2μg	18.10	8.74
4. 3—(1+2)	+1.27	−1.38
5. Purified beef heart lipid 2λ[b]	13.20	.....
6. Purified beef heart lipid 2λ plus standard α-Tocopherolquinone 2μg	13.11	9.98
7. 6—(5+2)	−0.1	−0.14

Column temperature: 215C, Cell temperature 240C, Cell voltage 1000 V with Strontium 90, Gain 3×10^{-9} AMP.

[a] Prior to silicic acid chromatography according to scheme II.

[b] After silicic acid chromatography.

Table II and Figure 3 show the gas chromatographic behavior of the tocopherols and their trimethylsilyl ethers. By the procedure described earlier, the silyl ethers of the free tocopherols are readily formed and have a shorter retention time than the parent compounds. Squalene, α-tocopherolquinone, and vitamin K_1 do not show any alteration in retention time. This is in keeping with the theoretical improbability of a silyl ether derivative.

Table III shows the effect of a crude beef heart muscle extract on authentic α-tocopherolquinone. When mixed with a crude lipid it could be seen that there is a reduction in peak area of α-tocopherolquinone and is associated with a concurrent rise in α-tocopherol. A quantitative relationship seems to

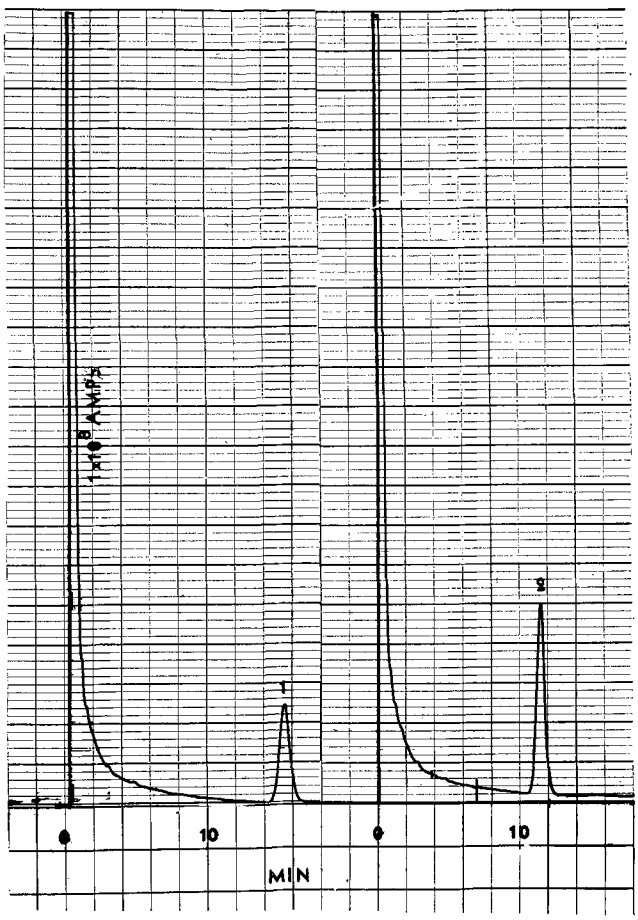
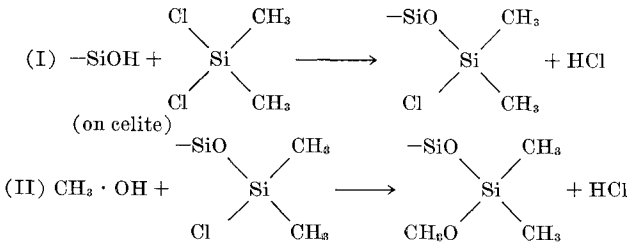


FIG. 4. Gas chromatographic detection of α-tocopherol from rat heart lipid. 1. Chromatography of untreated extract showing presence of α-tocopherol. 2. Chromatography of same sample as silyl derivative together with 0.5 μg standard trimethylsilyl α-tocopherol. Column temperature 240C; Flow rate 120 ml/min; Gain 1×10^{-8} AMP.

exist between the amount of original lipid taken to the amount of quinone converted to α-tocopherol. The lipid on passage through a silicic acid column is thereafter deprived of its capacity to carry out this conversion, apparently by the elimination of an unknown component. Figure 4 shows a typical chromatogram of rat heart lipid. Curve 1 shows the presence of α-tocopherol, and is confirmed in curve 2 by conversion to the corresponding silyl ether and mixed with authentic trimethylsilyl α-tocopherol.

In the preparation of the coated support for the column, there seems to be a number of chemical reactions involved. An appreciation of these chemical changes provide a clearer insight into phenomena connected with column performance. All commercially available diatomaceous earths have to be rigorously acid washed in the laboratory to ensure removal of heavy metal contaminants such as iron and chromium. The dry, acid washed support when treated with dimethyldichlorosilane and subsequently washed with methanol possibly undergoes a two stage chemical reaction as postulated below:



This type of chemical bonding and the formation of the active methoxy group on the siliconized support seems to be essential for the formation of chemically bonded liquid phase on the surface. Substitution of dimethyldichlorosilane with trimethylchlorosilane resulted in excessive bleeding of the phase, with a concomitant loss in performance. It is conceivable that there is an exchange reaction between the methoxy groups on the support and the silicone rubber polymers.

In this study two different liquid phases have been mixed together to form a two-component stationary phase in a gas-liquid chromatographic column. The functions of each of these two phases are as follows. The 5 moles % phenyl groups on SE 52, separate the isomers of the free tocopherols, but do not separate α-tocopherolquinone and α-tocopherolhydroquinone from α-tocopherol itself. The latter function is then performed by the nitrile polysiloxane rubber polymer of 287-108-949. By careful adjustment of the proportion of the two as also the percentage coating, it is possible to clearly separate α-tocopherol from its esters. An alternate technique would be to convert the free tocopherols to the trimethylsilyl ethers, leaving the esters unreacted.

The application of gas chromatography to the separation and determination of vitamins E and K, however do not preclude the necessity of initial purification of biological samples. It has to be specially emphasized that sterols and sterol esters, if present, can appear in the region of the tocopherols in the gas chromatogram. Biological extracts have to be rigorously purified to exclude compounds of this nature. Standards of Co-enzyme Q_{10}, vitamin A alcohol and vitamin A acetate do not appear in the chromatograms and are presumably retained on the column. Vitamin K and its analogues have retention times far removed from those of the vitamin E group. The positional isomers, β and γ tocopherol (5,8 dimethyltocol and 7,8 dimethyltocol) do not resolve in the gas chromatogram for unknown reasons.

The scheme in Figure 1 provides a means of checking the identity of the compounds indicated on the gas chromatograms. It could be seen that there are two alternate methods for initial purification. In each case an aliquot is directly chromatographed for vitamin E and K and the rest converted into the corresponding TMS derivatives and then chromatographed. Vitamin K comes out unchanged during this procedure. By careful collection of the effluent, the tocopherols could be recovered and tested chemically using the Emmerie-Engel reaction or the phosphomolybdic acid reaction of Nair and Magar (2). The potassium bromide pellet infrared spectra of the TMS α-tocopherol showed strong bands at 8.0, 10.58, and 11.2 and a medium band at 13.2μ. The 2.8μ band for hydroxyl group is absent, indicating the quantitative conversion of the tocopherol to the corresponding silyl ether.

The observation of a conversion of α-tocopherolquinone to α-tocopherol by crude biological extracts may have important implications and hence has to be carefully examined before a mechanism could be postulated. However, it could be safely surmised that there is an unknown component mediating in this reaction and which could be removed by chromatography through silicic acid. Then the very natural question would be to determine whether this conversion takes place only under the conditions of gas chromatography or whether this is a biological phenomenon. The former case is highly improbable considering the fact that an unidentified component is a limiting factor.

This brings us to the problems concerning the biological role of vitamin E, which will have to be reexamined in the light of the present findings. The antioxidant properties of vitamin E are very well known and have been the mainstay of extensive investigations into its biological role as a non-specific antioxidant. It is therefore natural to assume that part of the tocopherol gets oxidized to the quinone in the course of its action as an antioxidant. It is, however, interesting to note that during our investigations we have not been able to detect any α-tocopherolquinone in our biological samples. The high sensitivity of the technique together with its capacity to distinguish between its metabolites and oxidation products (α-tocopherolquinone) renders the biologist the means to clearly define the status of a biological sample.

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REFERENCES

1. Emmerie, A., and C. Engel, Rec. Trav. Chim., *57*, 1351–1355 (1938).
2. Nair, P. P., and N. G. Magar, J. Biol. Chem., *220*, 157–159 (1956).
3. Quaife, M. L., *Ibid.*, *175*, 605–617 (1948).
4. Edwin, E. E., A. J. Diplock, J. Bunyan, and J. Green, Biochem. J., *75*, 450–456 (1960).
5. Duggan, D. E., Arch. Biochem. Biophys., *84*, 116–122 (1959).
6. Seher, A., Mikrochim. Acta, 308–13 (1961), C. A. *55*, 14176f (1961).
7. Nicolaides, N., J. Chromatog. *4*, 496–499 (1960).
8. Simon, E. J., A. Eisengart, L. Sundheim, and A. T. Milhorat, J. Biol. Chem., *221*, 807–817 (1956).
9. Report of Vitamin E Panel, Soc. Anal. Chem., Quoted in Analyst *84*, 356–372 (1959).

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