Identification and Estimation of Tocopherols by Gas-Liquid Chromatography

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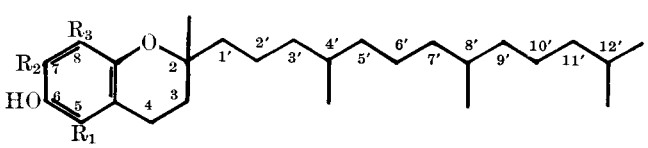
Abstract

A gas chromatographic method is described for the identification and estimation of the individual tocopherols as their trimethylsilyl (TMS) ethers. The TMS ethers are prepared by dissolving the sample in a mixture of hexamethyldisilazane, trimethylchlorosilane, and anhydrous pyridine (2:1:10), and allowing it to stand for at least 15 min. The separations described were made at 235C on 0.08 in. I.D. × 15 ft silanized glass columns packed with either 0.5% Apiezon L or 2% SE-30 on 110/120 mesh Anakrom (acid and base washed, and silanized). Retention data, obtained either by chromatographing known compounds or by prediction using the Kováts Retention Indices, are presented for tocol, tocotrienol, and all 14 possible methylated tocols and tocotrienols. The quantitative results from the analyses of two standard mixtures are also presented. The application to naturally occurring tocopherols is illustrated by chromatograms of partially purified fractions from soy oil, wheat germ oil, whole wheat flour, and corn meal.

Introduction

THE TENTATIVE RULES for naming the naturally-occurring tocopherols, recently adopted by the Commission on Biological Nomenclature of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (1), recognize and name eight naturally occurring tocopherols. These belong to two series of methyl-substituted chromanols with either a saturated [I] or an unsaturated [II] side chain in the 2-position.

I



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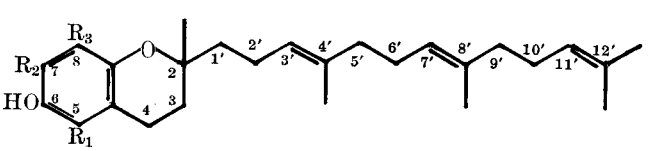

Fourteen different compounds are possible, bearing at least one methyl group on the aromatic ring. The parent unsubstituted compounds ($R_1=R_2=R_3=H$) tocol [I] and tocotrienol [II] have not been found in nature. The six possible tocopherols omitted from the new rules include the previously reported 7-methyltocol (2) and 5,7-dimethyltocol (3). Table I compares the old and new nomenclature. In this paper we have used the designations α, β, γ, δ, and named all other tocopherols as methyl derivatives of tocol or tocotrienol.

The determination of the individual tocopherols has posed a difficult analytical problem. A recent compilation of the technical literature on the vitamin E content of foods and feeds (4) lists 455 references, of which only 38 give data on the individual tocopherols. All the others give either total tocopherol content, or amounts of alpha and non-alpha. But since the tocopherols differ in vitamin potency (5), a knowledge of the individual forms is necessary for a correct estimation of the true vitamin content of a food or feed. The most successful method for the analysis of the individual tocopherols has employed two-dimensional paper chromatography for separation and identification, followed by a colorimetric estimation of the eluted spots (6). Because this method is slow, and the results sometimes difficult to interpret, it has not been widely used for the analysis of large numbers of samples.

Gas chromatography has also been investigated as a means for analyzing tocopherols. Wilson et al (7) chromatographed the seven tocols and two tocotrienols, both free and acetylated, on SE-30 and QF-1. On SE-30, δ and 7-methyltocol eluted together, as did the three dimethyltocols. 5,8-Dimethyltocotrienol was not separated from α. On QF-1, it was possible to separate the two tocotrienols. Quantitative estimates were said to agree well with those from two-dimensional paper chromatography. All the tocols were chromatographed on Apiezon N by Kofler et al (8). The separations of β from γ and of 7-methyltocol from 5-methyltocol were incomplete. No quantitative results were given. The other GLC investigations were of more limited scope. Nicolaides (9) was able to separate γ from α on silicone rubber gums. Bieri and Andrews (10) determined α-tocopherol on SE-30. α, β, and γ-Tocopherols and their TMS ethers were chromatographed on SE-52 and a mixed nitrile siloxane rubber by Nair and Turner (11). The dimethyltocols were not resolved. Libby and Sheppard (12)

TABLE I

Nomenclature of Tocopherols

R_1	R_2	R_3[a]	IUPAC-IUB designation [1]	Earlier designation
Structure I[a]				
H	CH_3	H	.....	η-tocopherol
H	H	CH_3	8-methyltocol (δ-tocopherol)	δ-tocopherol
CH_3	CH_3	H	.....	ζ_2-tocopherol
CH_3	H	CH_3	5,8-dimethyltocol (β-tocopherol)	β-tocopherol
H	CH_3	CH_3	7,8-dimethyltocol (γ-tocopherol)	γ-tocopherol
CH_3	CH_3	CH_3	5,7,8-trimethyltocol (α-tocopherol)	α-tocopherol
Structure II[a]				
H	H	CH_3	8-methyltocotrienol (δ-tocotrienol)	.....
CH_3	H	CH_3	5,8-dimethyltocotrienol (β-tocotrienol)	ϵ-tocopherol
H	CH_3	CH_3	7,8-dimethyltocotrienol (γ-tocotrienol)	.....
CH_3	CH_3	CH_3	5,7,8-trimethyltocotrienol (α-tocotrienol)	ζ_1-tocopherol

[a] See text.

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TABLE II

Retention Data for Tocopherol TMS Ethers[a]

Compound	Apiezon L		SE-30	
	Retention ratio (R_R) ($C_{28}H_{58}$ = 1.000)	Retention index (I_{235C})	Retention ratio (R_R) ($C_{28}H_{58}$ = 1.000)	Retention index (I_{235C})
Tocol	1.02–1.03	2807 ± 1.3	1.21	2867 ± 1.4
5-Methyltocol	1.42–1.44	2905 ± 1.1	1.60–1.62	2964 ± 2.5
7-Methyltocol	1.22–1.23	2860 ± 0.7	1.40–1.41	2917 ± 1.3
8-Methyltocol	1.03–1.05	2812 ± 1.8	1.29	2889 ± 0.0
5,7-Dimethyltocol	2.00–2.03	3002 ± 1.7	2.06–2.07	3052 ± 0.9
5,8-Dimethyltocol	1.37–1.38	2893 ± 1.0	1.65–1.66	2974 ± 1.2
7,8-Dimethyltocol	1.42–1.43	2904 ± 0.9	1.70–1.74	2987 ± 2.9
5,7,8-Trimethyltocol	2.31–2.34	3044 ± 1.7	2.42–2.49	3112 ± 2.9
Tocotrienol[b]	1.35	2889	1.60	2963
5-Methyltocotrienol[b]	1.91	2987	2.12	3060
7-Methyltocotrienol[b]	1.63	2942	1.85	3013
8-Methyltocotrienol[b]	1.38	2894	1.71	2985
5,7-Dimethyltocotrienol[b]	2.67	3084	2.72	3148
5,8-Dimethyltocotrienol	1.80–1.82	2972 ± 1.4	2.15–2.19	3069 ± 1.8
7,8-Dimethyltocotrienol[b]	1.90	2986	2.26	3083
5,7,8-Trimethyltocotrienol	3.08–3.12	3129 ± 1.3	3.22–3.24	3208 ± 1.1

[a] Instrument: modified F&M Model 810; on-column injection; carrier gas preheated. Electrometer normally operated at 1.6×10^{-11} amp. full scale. Columns: glass, silanized, ⅛ in. O.D. × 0.08 in. I.D. × 15 ft, packed with either 0.5% Apiezon L or 2% SE-30 on 110/120 mesh Anakrom, acid and base washed and silanized (Analabs, Incorporated). Carrier gas: Helium, 15–20 ml/min; tank pressure 60 psig. Temperatures: oven, 235C; injection, 235C; detector 265C.

[b] Calculated, using the Retention Index Increment of the side-chain unsaturation. See text.

have developed a quantitative method for free and acetylated α, γ, and δ tocopherols using SE-30 columns.

The present work was undertaken in an effort to develop a specific assay suitable for routine analysis for the individual tocopherols in wheat. The chromatographic separation of the free tocopherols on Apiezon L was not wholly successful, due to variable sample loss on the column and tailing peaks. The TMS ethers, prepared by the method of Sweeley et al. (13) proved much more satisfactory. The choice of stationary phases was necessarily limited to those stable at high temperatures, and was further restricted to those giving at least a partial separation of β and γ. No available stationary phase more polar than SE-30 met these criteria.

Procedures

Materials

α, β, γ, and δ-Tocopherols: Distillation Products Industries.

5,7-Dimethyltocol, 5,8-dimethyltocotrienol, and 5,7,8-trimethyltocotrienol: Hoffmann-La Roche, Inc.

Tocol: Pierce Chemical Company.

5-Methyltocol and 7-methyltocol: Synthesized by the condensation of methylhydroquinone and phytol (14,15). The mixture of 5-, 7-, and 8-monomethyltocols was separated by thin-layer chromatography (TLC) (8) and the spots corresponding to 5-methyltocol and 7-methyltocol scraped off, eluted with diethyl ether, and chromatographed as their TMS ethers. Both spots gave positive reactions when sprayed with ferric chloride/α, α′-dipyridyl, antimony pentachloride and phosphomolybdic acid. The 8- and 7-methyltocol spots coupled with diazotized o-dianisidine: the 5-methyltocol spot did not. The 8-methyltocol in the mixture was identified as δ both by TLC and GLC.

Hexamethyldisilazane and trimethylchlorosilane: Applied Science, Inc.

Pyridine, anhydrous: distilled and stored over KOH pellets.

Octacosane ($C_{28}H_{58}$): Eastman white label.

Preparation of Trimethylsilyl Ethers

The sample, containing 25 to 50 μg of each tocopherol, was dissolved in 0.1 ml of a solution consisting of 10 parts of anhydrous pyridine, 2 parts of hexamethyldisilazane, and 1 part of trimethylchlorosilane. After standing at room temperature for at least 15 min, the mixture was injected by syringe onto the gas chromatograph without further treatment. The amount injected varied from 0.2 to 1.0 μl, containing 0.05 to 0.5 μg.

Results and Discussion

Retention Data

Each available tocopherol was mixed with octacosane ($C_{28}H_{58}$), derivatized and chromatographed. Their retention ratios (R_R) relative to octacosane are given in Table II. Retention times were measured from the solvent peak. The Kováts Retention Index [I] (16) was also calculated, based on the complete series of both odd and even straight chain hydrocarbons. The Retention Index, which has been proposed as a standard method of reporting retention data, may be used for predicting retentions by means of the constant Retention Index Increments [ΔI] of specific molecular features. It has been used here to estimate the Retention Indices and R_R of the unavailable tocotrienols. The increase in retention due to the unsaturation in the side chain was calculated as the difference between the Retention Indices of α and β and their unsaturated analogs 5,7,8-trimethyltocotrienol and 5,8-dimethyltocotrienol. On Apiezon L the two values obtained were 85 and 79 (ave = 82); on SE-30, 96 and 95 (ave = 96). This ΔI (unsaturation) was added to the Retention Indices of the appropriate tocols to give the Retention Indices of the related tocotrienols (Table II). The ΔI of the aromatic methyl groups were inconsistent. On Apiezon L, for example, the ΔI of the 5-methyl group is 81 if taken as the difference between 8-methyltocol (δ) and 5,8-dimethyltocol (β), and 140 if calculated as the difference between 7,8-dimethyltocol (γ) and 5,7,8-trimethyltocol (α). Similar discrepancies may be noted by comparing values in Table II.

Complete separation of all the tocopherols was not possible on either phase. Typical chromatograms of a synthetic mixture, containing seven tocopherols, are shown in Fig. 1. The most serious interference was that between β and γ. Apiezon L gave the best separation for this pair, and was chosen for the quantitative estimations. However, if one peak were very small it would be difficult to measure. Both phases might be expected to separate them equally well, since the separation factors are approximately the same, but on SE-30 the two peaks are shifted toward one another. There are also other interferences, notably that between 8-methyltocotrienol and either β (Apiezon L) or γ (SE-30). These uncertainties may be resolved by a preliminary thin-layer chromatographic fractionation into groups separable by GLC. But the tocopherols reported in foods should not require this treatment.

Although retention ratios were reproducible on different columns, the efficiencies of otherwise similar columns varied widely. This was no doubt partially due to the difficulty of preparing consistent low-loaded packings. Careful silanization of both the support

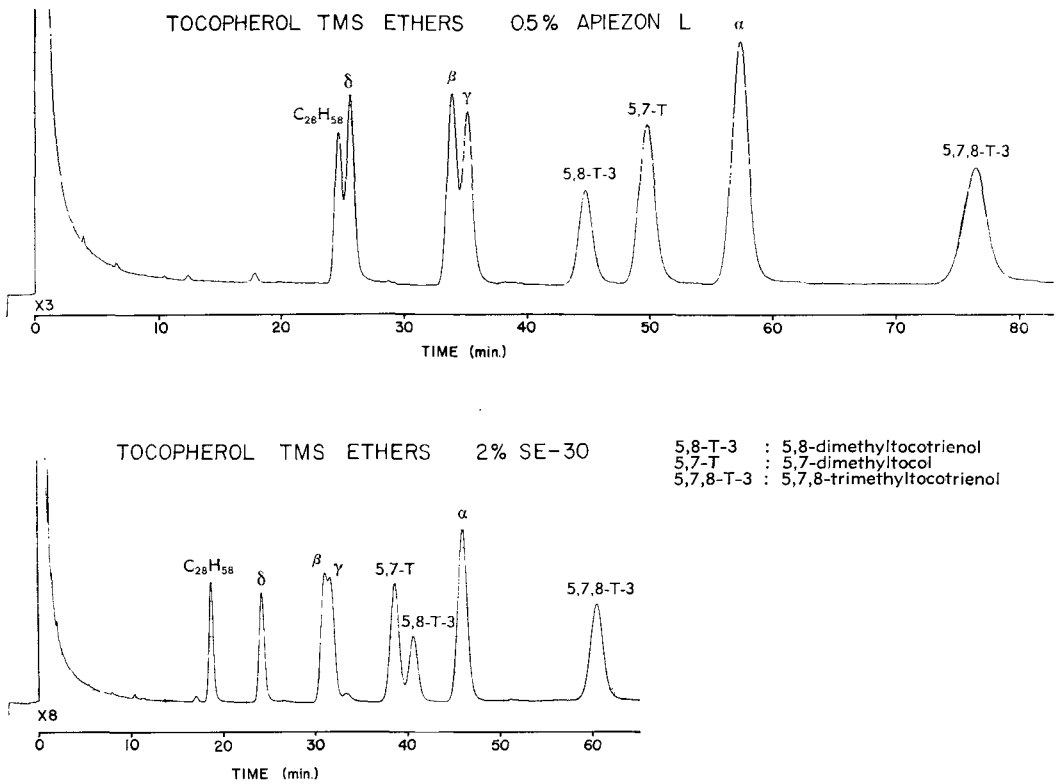

FIG. 1. Separation of trimethylsilyl ethers of a mixture of known tocopherols on Apiezon L and SE-30.

material and the glass column was essential to good column performance. Columns were conveniently silanized by filling them with the silylating reagent, allowing them to stand for an hour, then washing them thoroughly with anhydrous methanol. The columns used in this study had efficiencies of 400-450 theoretical plates per foot.

Quantitative

Two mixtures of tocopherols, containing octacosane as an internal standard, were used to assess precision and accuracy. The normalized area percentages obtained from five chromatograms of each mixture are given in Table III. Areas were calculated as the product of peak height and retention time. This method was chosen in order to cope with the incompletely resolved β and γ peaks. Because the tocopherol samples used were not completely free of other tocopherols, each sample was analyzed separately by GLC and the calculated composition used to determine the "weight percent (corrected)" given in Table III. Some decomposition doubtless occurred on the column and seemed to be related to residence time as well as to the high temperature. This effect of residence time was indicated by the coincidence of poor tocopherol recovery and long retention times with samples chromatographed either at lower temperatures on 15 ft columns or at 235C on longer columns. The conditions used were a compromise chosen to give the best resolution with minimum decomposition. For the analysis of simpler mixtures shorter columns and lower temperatures could be used to reduce tocopherol loss.

Under the conditions used, sensitivity varied from approximately 4×10^{-9} g of δ to 12×10^{-9} g of 5,7,8-

TABLE III

Quantitative Data on Known Mixtures of Tocopherols[a]

	α	β	γ	δ	5,7-dimethyltocol	5,8-dimethyltocotrienol	5,7,8-trimethyltocotrienol	$C_{28}H_{58}$
Standard No. 1								
Found:	18.2	14.3	.....	.....	39.3	13.2	.....	15.0
	17.1	14.4	.....	.....	38.3	12.8	.....	17.4
	18.2	13.7	.....	.....	38.4	14.5	.....	15.2
	18.8	13.5	.....	.....	35.0	12.8	.....	15.9
	19.2	13.3	.....	.....	39.0	14.7	.....	14.0
Mean	18.3	13.8			38.0	13.6		15.5
S.E.	±0.70	±0.44			±1.55	±0.83		±1.13
Wt. %, (corrected)	18.3	13.6			39.3	16.5		13.2
Standard No. 2								
Found:	18.3	7.0	13.0	15.1	16.2	7.8	6.3	16.3
	17.8	7.2	13.0	14.4	16.8	8.4	6.7	15.6
	17.8	7.1	13.0	15.1	16.1	7.7	6.1	16.7
	19.2	6.9	13.6	14.6	15.6	7.7	5.9	16.6
	19.7	7.2	13.6	13.4	16.5	7.4	5.4	16.8
Mean	18.6	7.1	13.2	14.5	16.2	7.8	6.1	16.4
S.E.	±0.77	±0.37	±0.28	±0.62	±0.40	±0.33	±0.43	±0.43
Wt % (corrected)	17.4	7.4	11.6	13.5	17.9	10.8	7.4	14.1

[a] Normalized area percent. All samples run on 0.5% Apiezon L. The chromatographic conditions are given in Table II.

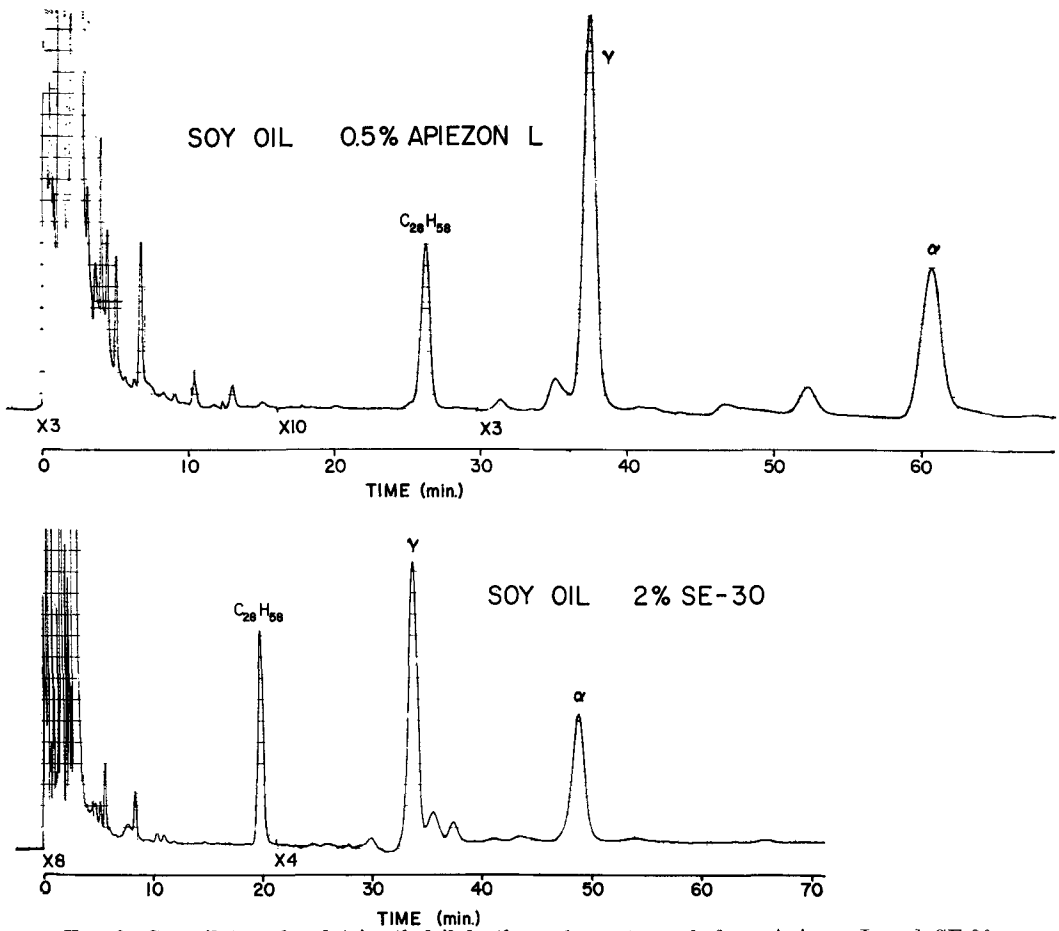

FIG. 2. Soy oil tocopherol trimethylsilyl ethers chromatographed on Apiezon L and SE-30.

trimethyltocotrienol, estimated as the weight required to give a signal twice the average noise level.

Application

The method was applied to the qualitative analysis of a few natural tocopherol mixtures. Quantitative estimations are not reported because the extraction and purification procedures used have not been investigated in detail. Each type of sample will doubtless present its own unique problems. The techniques necessary for the quantitative extraction and pur-

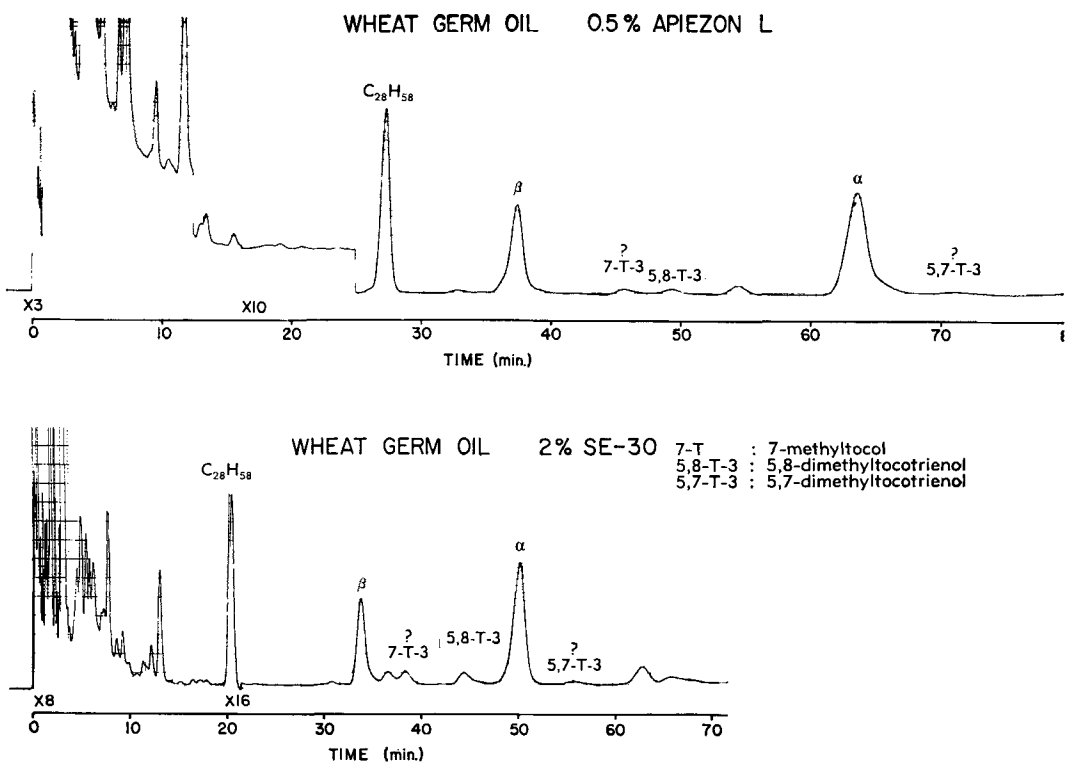

FIG. 3. Wheat germ oil tocopherol trimethylsilyl ethers chromatographed on Apiezon L and SE-30.

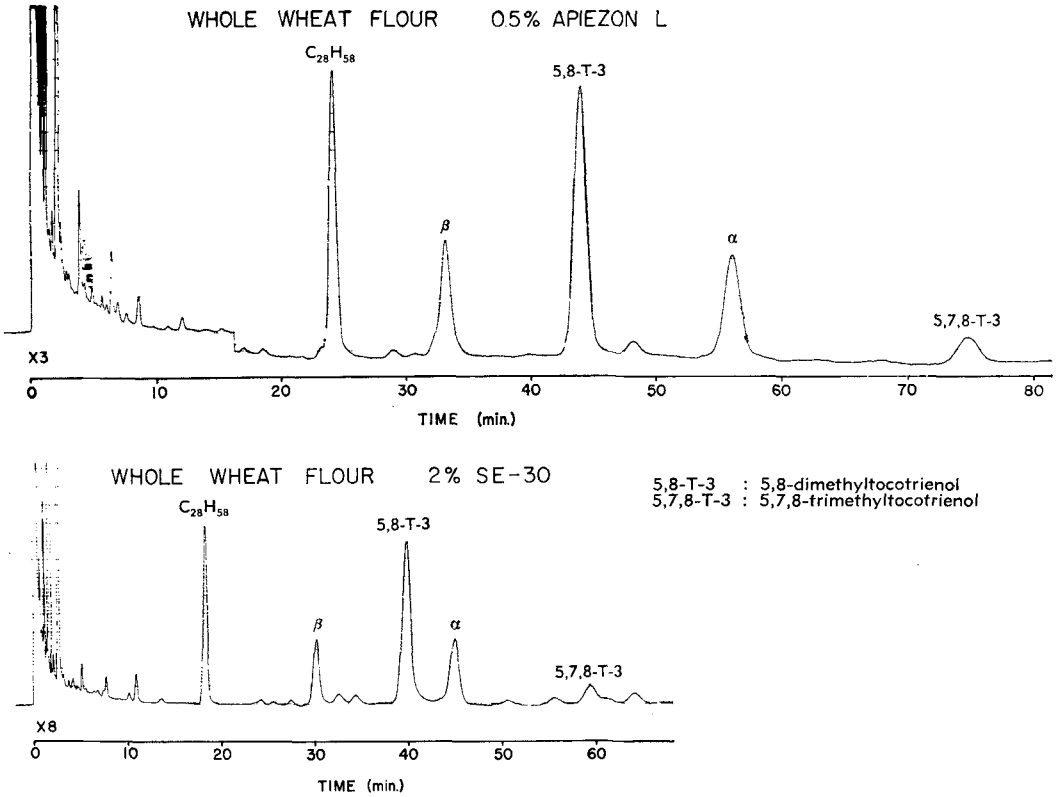

FIG. 4. Whole wheat flour tocopherol trimethylsilyl ethers chromatographed on Apiezon L and SE-30.

ification of tocopherols from wheat and wheat products are now being investigated in this laboratory.

Each sample was chromatographed on each of the two phases. Only those peaks identifiable on both Apiezon L and SE-30 as tocols or tocotrienols are mentioned. The identities of the previously reported tocopherols are considered reasonably valid, but those of the unreported or controversial compounds are at best tentative and require further characterization.

Soy oil (Fig. 2). One gram of commercial soy oil was saponified under N_2, with pyrogallol added. The nonsaponifiable fraction was extracted with pentane, passed through Florosil (17) and separated by TLC on Silica Gel G. The tocopherol area was scraped off, derivatized and chromatographed. Only α and γ tocopherols were found. δ-Tocopherol should also be present (4) but was possibly lost on the Florosil column.

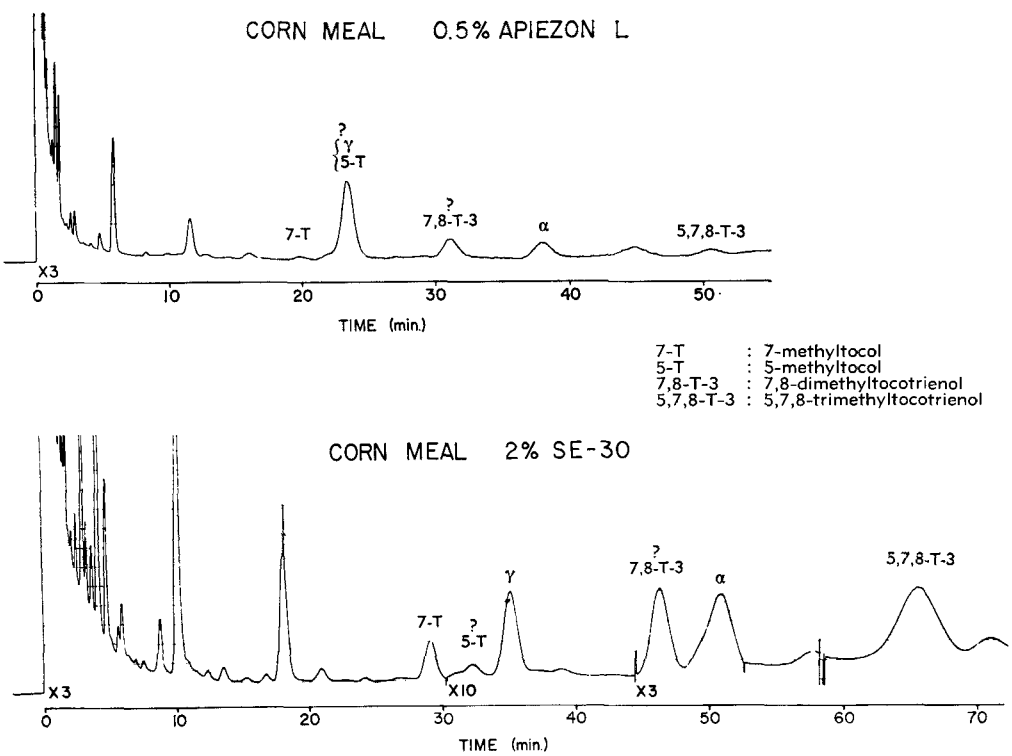

FIG. 5. Corn meal tocopherol trimethylsilyl ethers chromatographed on Apiezon L and SE-30.

Wheat germ oil (Fig. 3). One gram of wheat germ oil was processed in the same way as was the soy oil. The principal tocopherols found were α and β. The small amount of 5,8-dimethyltocotrienol is to be expected from the published data. Minor peaks with retention ratios corresponding to those estimated for 7-methyltocotrienol and 5,7-dimethyltocotrienol were also found.

Whole Wheat Flour (Fig. 4). Whole wheat flour was Soxhlet extracted for 20 hr with absolute ethyl alcohol, and the nonsaponifiable fraction purified by TLC. No Florosil clean-up was used. The peaks identifiable as tocopherols were α, β, 5,8-dimethyltocotrienol, and 5,7,8-trimethyltocotrienol. These identifications conform to the published data obtained by the use of two-dimensional paper chromatography.

Corn meal (Fig. 5). The sample was processed in the same way as the whole wheat flour. This sample contained no internal standard: octacosane was chromatographed just before injecting the sample. In addition to the previously reported α and γ, peaks with the retention ratios of 7-methyltocol, 7,8-dimethyltocotrienol, and 5,7,8-trimethyltocotrienol were found.

ACKNOWLEDGMENT

Samples of 5,7-dimethyltocol, 5,8-dimethyltocotrienol, and 5,7,8-trimethyltocotrienol were a gift from Hoffmann-La Roche, Inc.

REFERENCES

1. Anon., Biochim. Biophys. Acta *107*, 1–4 (1965).
2. Green, J., and S. Marcinkiewicz, Nature *176*, 86–7 (1956).
3. Green, J., D. McHale, S. Marcinkiewicz, P. Mamalis and P. R. Watt, J. Chem. Soc. *1959*, 3362–3373.
4. Dicks, M. W., "Vitamin E Content of Foods and Feeds For Human and Animal Consumption," Bul. 435, Wyo. Agric. Exp. Sta., Univ. of Wyo. 1965.
5. Bunyan, J., D. McHale, J. Green and S. Marcinkiewicz, Brit. J. Nutr. *15*, 253–257 (1961).
6. Green, J., S. Marcinkiewicz and P. R. Watt, J. Sci. Food Agr. *6*, 274–282 (1955).
7. Wilson, P. W., E. Kodicek and V. H. Booth, Biochem. J. *84*, 524–531 (1962).
8. Kofler, M., P. F. Sommer, H. R. Bolliger, B. Schmidli and M. Vecchi, "Vitamins and Hormones," Vol. 20, Academic Press Inc., New York, 1962, p. 407–439.
9. Nicolaides, N., J. Chromatog. *4*, 496–499 (1960).
10. Bieri, J. G., and E. L. Andrews, Iowa State J. Sci. *38*, 3–12 (1963).
11. Nair, P. P., and D. A. Turner, JAOCS *40*, 353–356 (1963).
12. Libby, D. A., and A. J. Sheppard, J. Am. Offic. Agric. Chem. *47*, 371–376 (1964).
13. Sweeley, C. C., R. Bentley, M. Makita and W. W. Wells, J. Am. Chem. Soc. *85*, 2497–2507 (1963).
14. Karrer, P., and H. Fritzsche, Helv. Chim. Acta *22*, 260–263 (1939).
15. Marcinkiewicz, S., D. McHale, P. Mamalis and J. Green, J. Chem. Soc. *1959*, 3377–3378.
16. Ettre, L. S., Anal. Chem. *36*, 31A–41A (1964).
17. Analytical Methods Committee: Vitamin E Panel, Analyst *84*, 356–372 (1959).

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