Vitamin E in Foods: Determination of Tocols and Tocotrienols

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Abstract

A method is described for the analysis of foods for the forms of vitamin E. Detailed procedures are given for extraction, saponification and partial purification by thin layer chromatography. The individual tocopherols (both tocols and tocotrienols) are identified and estimated as their trimethylsilyl ethers by gas liquid chromatography on SE-30 or Apiezon L at 235 C. Retention ratios are also given for separations on OV-17. Response factors relative to didecyl pimelate as an internal standard and overall recoveries were determined for α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, β-tocotrienol and α-tocotrienol. Sample sizes depended on tocopherol content and were usually chosen to contain 3–50 μg of the individual tocopherols. Data for a number of seeds and oils are given. The greatest variety of forms was found in barley, which contains all the forms listed above, plus γ-tocotrienol.

Introduction

The analysis of foods for vitamin E requires a method capable of distinguishing its various forms. Eight of the 14 methyl derivatives of tocol [2-methyl-2(4′,8′,12′-trimethyltridecyl)chroman-6-ol] and tocotrienol [2-methyl-2(4′,8′,12′-trimethyltrideca-3′,7′,11′-trienyl) chroman-6-ol] are now recognized as naturally occurring tocopherols (1). All of these compounds have been found to differ widely in both in vivo vitamin E potency (2) and antioxidant activity (3). Although a few foods may contain only a single tocopherol, most contain a variety of forms whose individual amounts must be known if a true calculation of vitamin E content is needed.

A recent publication from this laboratory (4) described a gas chromatographic analysis of synthetic mixtures of tocopherols, and gave retention data on SE-30 and Apiezon L for the trimethylsilyl (TMS) ethers of all the methylated tocols and tocotrienols. This communication concerns the extension of that work to the analysis of tocopherols in foods. Detailed procedures for tocopherol extraction and purification have been devised that minimize losses of the easily oxidized vitamin and decrease the number of interfering substances.

The nomenclature used in this paper (Table I) conforms to the recent Tentative Rules adopted by the IUPAC (1). α-T-3 has also been known as ζ_1-tocopherol; β-T-3 as ε-tocopherol.

Materials

The following materials were used: α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol, from Distillation Products Industries; α-tocotrienol, β-tocotrienol and 5,7-dimethyltocol, from Hoffmann-La Roche Inc.; 5-methyltocol and 7-methyltocol, synthesized from phytol and methylhydroquinone (4); and γ-tocotrienol and δ-tocotrienol, separated by preparative gas liquid chromatography from a fraction of the unsaponifiable lipid from NH_3-stabilized concentrated latex emulsion (5). Other materials used were hexamethyldisilazane and trimethylchlorosilane (Pierce Chemical Company), pyrogallol (reagent grade) and didecyl pimelate (mp 25–26C) synthesized from decyl alcohol and pimelic acid.

Solvents subjected to extensive concentration were tested by concentrating 200 ml of 0.1 ml, then injecting 2 μl of the concentrate onto an analytical column. Those giving peaks other than the solvent peak were purified further.

Absolute ethanol was distilled over acidified 2,4-dinitrophenylhydrazine and then redistilled. Anhydrous pyridine was distilled and stored over KOH pellets. Peroxide free ethyl ether was used without further purification; ether in cans was siphoned off, since the surface of the cans contributes an ether soluble contaminant. Benzene (reagent grade) and anhydrous methanol (reagent grade) were used without further treatment. Petroleum ether (30–60 C) was distilled if necessary, but some commercial products were found of adequate purity.

Experimental Procedures

All work was performed under subdued light provided by gold fluorescent lights with a cut-off below 5000 A and above 7600 A. Sample sizes, depending on tocopherol content, ranged from 5 to 20 g for solid samples and from 0.025 to 4 g for fats and oils. Best results were obtained with samples containing from 2 to 200 μg of each tocopherol.

TABLE I
Relative Retention Data for the Tocopherol TMS Ethers[a]

Compound	Abbreviation	SE-30	Apiezon L	OV-17
Tocol	T	0.69	0.67	0.55
5-Methyltocol	5-T	0.91	0.93	0.74
7-Methyltocol	7-T	0.79	0.79	0.62
8-Methyltocol (δ-tocopherol)	δ-T	0.73	0.68	0.56
5,7-Dimethyltocol	5-7-T	1.16	1.33	1.01
5,8-Dimethyltocol (β-tocopherol)	β-T	0.93	0.91	0.71
7,8-Dimethyltocol (γ-tocopherol)	γ-T	0.95	0.93	0.73
5,7,8-Trimethyltocol (α-tocopherol)	α-T	1.37	1.53	1.14
Tocotrienol[b]	T-3	0.91	0.89	0.98
5-Methyltocotrienol[b]	5-T-3	1.21	1.26	1.32
7-Methyltocotrienol[b]	7-T-3	1.05	1.07	1.12
8-Methyltocotrienol (δ-tocotrienol)	δ-T-3	0.95	0.90	1.00
5,7-Dimethyltocotrienol[b]	5,7-T-3	1.55	1.76	1.78
5,8-Dimethyltocotrienol (β-tocotrienol)	β-T-3	1.21	1.19	1.27
7,8-Dimethyltocotrienol (γ-tocotrienol)	γ-T-3	1.25	1.24	1.33
5,7,8-Trimethyltocotrienol (α-tocotrienol)	α-T-3	1.81	2.04	2.05
Octacosane		0.57	0.66	0.29
Didecyl pimelate[c]	DDP	1.00	1.00	1.00

[a] Instrument: F&M Model 810 with flame ionization detector; on-column injection; electrometer normally operated at 1.6 × 10^{-11} amp. full scale. Columns: glass, silanized, 0.125 in. O.D. × 0.08 in. I.D. × 15 ft. Packing: Apiezon L, 0.4%; SE-30, 1.7%; OV-17, 1.6%; on Gas Chrom Q 100/120. Carrier gas, helium 30 ml/min. Oven 235 C; injection port 235 C; detector 265 C.
[b] Calculated from Kováts Retention Indices (4).
[c] Retention time in minutes of DDP (approx.); SE-30, 50; Apiezon L, 26; OV-17, 64.

Extraction

Solid foods were ground for three 30 sec intervals in a water-cooled high speed micromill. The weighed sample was placed in a 500 ml glass-stoppered low actinic Erlenmeyer flask and exactly 150 ml of anhydrous ethanol added, along with 0.2 g of pyrogallol. The flask was flushed with nitrogen for 5 min, stoppered and shaken at room temperature on a mechanical shaker for 1 hr at a speed just fast enough to keep all the sample suspended. The extract was filtered through fluted filter paper (Whatman No. 43), without washing the residue on the filter. An aliquot of the filtrate (100 ml or less) was taken for analysis. This treatment of the extract avoided the need for its quantitative recovery. The effective sample weight (W_S) was given by: volume of aliquot times weight of sample extracted divided by 150.

Saponification and Thin Layer Chromatography

All samples, both lipid extracts from solids as well as fat and oil samples, were saponified in 100 ml of absolute ethanol to assure uniformity of treatment. The sample in ethanol, 2 ml of 10% alcoholic pyrogallol and a Teflon boiling chip were placed in a 500 ml low actinic Erlenmeyer flask fitted with an additive adapter bearing a 50 ml separatory funnel and a reflux condenser. An inert atmosphere was maintained in the flask by passing a slow stream of nitrogen in through the separatory funnel, interrupting the flow only to make additions through the funnel. After flushing the flask for 5 min, 5 ml of saturated aqueous KOH was added and the flask brought to a boil. After refluxing for 5 min, the heat source was removed and replaced by an ice bath. When the flask was cold, 100 ml of petroleum ether was added through the separatory funnel, followed by 100 ml of cold boiled distilled water. The flask was disconnected from the apparatus, quickly stoppered, shaken for 2 min, and the contents quantitatively transferred to a 500 ml separatory funnel. The lower water layer was drawn off into a second separatory funnel and re-extracted with 100 ml of petroleum ether. The two extracts were pooled and washed four times with 100 ml portions of cold boiled distilled water, adding 2 ml of 10% pyrogallol each time. Before shaking with the last water wash, a known amount of internal standard, didecyl pimelate (DDP), usually about 50 μg, was added. All the last water wash was drawn off, sacrificing a small amount of the petroleum ether solution when necessary. The extract was evaporated into the tip of a 100 ml Goetz centrifuge tube with a slow steam of nitrogen in a water bath at 45 C. The sides of the tube were rinsed down twice with ca. 3 ml of ethyl ether, which was also evaporated away. The residue was dissolved in 0.2 ml of benzene.

The entire benzene solution was separated on one 200 × 200 mm thin layer plate coated with a 0.25 mm layer of Silica Gel HF_{254} (Brinkmann) which contains a fluorescing indicator. A 1 in. panel along one side was spotted with a mixture of δ-T and α-T to define minimum and maximum tocopherol travel. The plate was covered with a second plate separated by 1/16 in. Teflon spacers along the sides and developed with benzene-methanol (98:2) for 15 cm. Tocopherols in the reference strip appeared as fluorescence-quenching spots under ultraviolet light. The tocopherol band defined by the reference spots was scraped from the plate and transferred to a 1.2 × 15 cm percolation tube with a plug of glass wool in the lower end. The tocopherol fraction was eluted with 2 × 10 ml of ethyl ether into a 50 ml glass-stoppered conical centrifuge tube and the ether removed with a nitrogen stream in a water bath at 45 C. After rinsing the sides of the tube with ether and evaporating the ether, the sample was ready for derivatization.

TABLE II

Relative Detector Response of Tocopherol TMS Ethers

Tocopherol	Relative response
Didecyl pimelate	1.00
α-T	1.43
β-T	1.45
γ-T	1.35
δ-T	1.45
α-T-3	1.30
β-T-3	1.25

Derivatization and Gas Liquid Chromatography

TMS ethers were made by adding 0.1 ml of a mixture of hexamethyldisilazane-trimethylchlorosilane-anhydrous pyridine (9:6:10). After standing at least 15 min the sample was injected onto a gas liquid chromatography (GLC) column without further treatment. Details of the separation are essentially as described earlier (4), and are given in Table I. Column packings were prepared by a modification of the solution coating technique (6). Twenty grams of the support was mixed with 100 ml of a solution of the liquid phase (0.5% Apiezon L in isooctane, 2% SE-30 in isooctane, or 2% OV-17 in toluene), stirred, filtered through a fritted funnel under vacuum and dried by drawing air through the packing. The percentage of liquid phase on these packings was slightly lower than the solution concentration. Solutions of 2% SE-30 gave phase loadings of approximately 1.7%, as determined both gravimetrically and by measuring the volume of solution remaining on the packing. The packings prepared with 2% OV-17 solutions were approximately 1.6%; those prepared with 0.5% Apiezon L were approximately 0.4%.

Calculation

Areas were calculated as base width times peak height divided by 2. All base widths in the same chromatogram were measured using portions of the peak at the same attenuation, since the base widths of peaks at different attenuations are not comparable.

The amount of each tocopherol was calculated as:

$$\frac{\text{mg tocopherol}}{\text{100 g sample}} = \frac{K_T \times A_T \times W_R \times 100}{A_R \times W_S}$$

Where: K_T = correction factor (Table V); A_T = area of tocopherol peak; W_R = weight of internal standard added (mg); A_R = area of internal standard peak; and W_S = sample weight (g).

TABLE III

Comparison of Extraction Methods: Recovery of Tocopherols Added to White Wheat Flour

Method	Recovery, per cent		
	Replicate	β-T-3	α-T
Soxhlet, 20 hr	1	5.8	51.8
	2	29.3	52.5
	3	62.6	68.0
Shaking, 20 hr	1	59.3	88.0
	2	83.1	82.6
Shaking, 1 hr	1	92.0	88.2
	2	97.1	87.8

TABLE IV

Recovery of Tocopherols From Entire Procedure (Per cent)

Tocopherol	Standard alone	Standard added to bread	Standard added to wheat bran
α-T	88	84	89
β-T	92	90	91
γ-T	89	....	....
δ-T	89	55	85
α-T-3	89	87	89
β-T-3	93	....	....

TABLE V

Tocopherol Correction Factors (K) for Correcting Areas for Response Variations and Analytical Losses

Tocopherol	K
α-T	0.80
β-T	0.75
γ-T	0.83
δ-T	0.78
α-T-3	0.86
β-T-3	0.86

Results and Discussion

One or more preliminary analyses were usually necessary to determine the qualitative composition, the optimum sample size and the best phase for the quantitative estimation. Identifications were based on retention behavior on all three phases (Table I), but only Apiezon L and SE-30 were used for quantitative work. OV-17 was not used for this purpose because tocotrienol retention times were longer than on the other two phases. None of the columns described could completely separate β-T from γ-T, although these forms could be estimated together on Apiezon L if the amount of one did not greatly exceed that of the other. There was no difficulty in identifying the major peak as either β-T or γ-T, however dissimilar their concentrations. The principal criterion for the choice of a phase for quantitation was its ability to separate tocopherol peaks from nontocopherol peaks, rather than its ability to separate β-T from γ-T.

The internal standard technique minimizes the necessity for quantitative transfers and dilutions and avoids the need for injecting known volumes onto the GLC columns. For some samples one of the tocopherols might serve, but for general use didecyl pimelate has been most satisfactory. It must be added after the saponification step, but thereafter quantitative transfers are not required. DDP is not affected by the silylating reagent and may be chromatographed without decomposition.

Small amounts of water interfered in derivatizations with solutions of hexamethyldisilazane-trimethylchlorosilane-pyridine (2:1:10). The removal of the last traces of water required drastic or lengthy treatment damaging to the samples. A silylating mixture with a reagent ratio of 9:6:10 was equally effective in forming TMS ethers, even in the presence of added water. Up to 5 μl of water has been added to 0.1 ml of this reagent without impairing the results. The derivatives were formed rapidly and were stable at room temperature over a period of hours. Although some preparations were stable for several days, all samples for quantitative determinations were derivatized and analyzed the same day.

Dimethylsilyl ethers have also been chromatographed, but were less stable and harder to separate than the corresponding TMS derivatives.

The relative responses of the TMS ethers of the tocols were slightly higher than those of the tocotrienols (Table II). These differences represent not only variations in specific detector responses but also losses on the column and during derivatization. The values were reproducible on different instruments provided with similar columns; this may not be true under other chromatographic conditions. The problems of response linearity were minimized by limiting acceptable tocopherol peaks to those with peak heights less than ca. 5 times that of the reference peak. Linearity well beyond this range was demonstrated for α-T and δ-T by derivatizing and chromatographing samples containing varying amounts of the tocopherol and a fixed amount of DDP (Fig. 1).

The extraction method described was chosen for its reliability and simplicity. Soxhlet extraction with

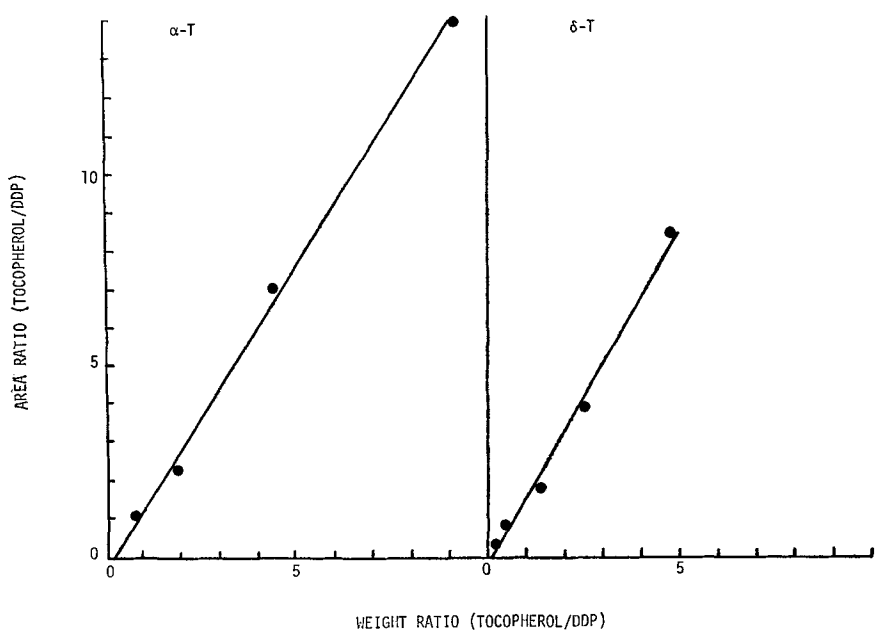

FIG. 1. Response linearity of α-T and δ-T. Sample size: α-T, 0.4 μg to 4 μg; δ-T, 0.1 μg to 2 μg; DDP, 0.5 μg; column: 15 ft × 0.08 in I.D., glass, packed with 1.7% SE-30 on Gas Chrom Q (100/120); carrier gas: Helium; and temperatures: injection port, 235 C, oven, 235 C, detector, 260 C.

TABLE VI

Tocopherols in Wheat Bran (10 Replicates)

Tocopherol	Amount found μg/g	Coefficient of variation
α-T	16.3 ± 0.81	5.0
β-T	10.1 ± 0.58	5.7
α-T-3	11.0 ± 0.81	7.3
β-T-3	53.7 ± 0.30	0.6

ethanol failed to give good recoveries of standards added to white wheat flour before extraction (Table III). Shaking for 1 hr gave better recoveries than either overnight shaking or overnight Soxhlet extraction. Efficiency of extraction was assessed by extracting representative samples twice and analyzing both extracts. No tocopherol was found in the second extract from flour or baked products. The first and second extracts from wheat grain contained (1 mg tocopherol/100 g sample); α-T, 1.3 and 0.1; β-T, 0.8 and 0.02; α-T-3, 0.3 and 0.05; and β-T-3, 2.8 and 0.2.

The recovery of standards submitted to the entire procedure, both alone and when added to foods, was determined by comparing relative area responses of identical samples with and without such treatment (Table IV). Approximately 10% of all forms was lost. The low return of δ-T added to bread was attributed to the large amount of unsaponifiable material in the extract, which interfered with the thin layer separation. In order to simplify calculations, correction factors (K) were determined for six of the eight tocopherols, to correct for analytical losses and for differences in response (Table V). It is equal to the reciprocal of the area response of tocopherols submitted to the entire procedure, relative to didecyl pimelate added after the saponification step. The two tocopherols for which response and correction factors are not given, δ-tocotrienol and γ-tocotrienol, are not widely distributed. γ-Tocotrienol in barley, fresh corn and coconut oil was calculated using the correction factor for β-tocotrienol.

Results from 10 analyses of another sample of bran, run by two operators on two different columns (Table VI) indicate that for this material at least the method is reasonably reproducible. Coefficients of variation varied from 0.6% to 7.3%; the higher values are more characteristic.

A few analyses of tocopherol-containing sources are given in Table VII. Most of these samples were bought in local stores and are of unknown history. The results are given here only to illustrate the applicability of the method. The tocopherol content of barley is especially interesting. It has all the naturally-occurring forms except δ-T-3, and is one of the few sources containing γ-T-3. The latter form

TABLE VII

Tocopherols in Seeds and Oils (mg/100 g fresh weight)

Sample	α-T	β-T	γ-T	δ-T	α-T-3	β-T-3	γ-T-3
Seeds							
Alfalfa	33.0	Trace	0.9	.....	.....	....	....
Almond (meal)	31.7	0.3	0.9	.....	0.5	....	....
Barley	0.2	0.04	0.03	0.01	1.1	0.3	0.2
Corn (fresh)	0.06	.....	0.4	.....	0.2	....	0.4
Millet	0.05	Trace	1.3	0.4	.....	....	....
Oats	0.5	0.09	.....	.....	1.1	0.2	....
Poppy	1.8	.....	9.2	.....	.....	....	....
Rye	1.6	0.4	.....	.....	1.5	0.8	....
Sesame	.....	.....	22.7	.....	.....	....	....
Wheat	1.0	0.7	.....	.....	0.4	2.8	....
Fats and oils							
Coconut	0.5	.....	.....	0.6	0.5	0.1	1.9
Corn-A	7.9	.....	44.7	0.4	.....	....	....
Corn-B	16.2	.....	60.3	.....	.....	....	....
Cottonseed	32.0	.....	31.3	.....	.....	....	....
Lard	1.2	.....	0.07	.....	0.07	....	....
Olive	0.8	.....	.....	.....	.....	....	....
Peanut	18.6	.....	13.8	.....	.....	....	....
Safflower	34.2	.....	7.1	.....	.....	....	....
Shortening, processed vegetable	9.9	.....	66.2	23.0	.....	....	....
Soybean-A	4.2	.....	25.2	5.3	.....	....	....
Soybean-B	9.4	.....	63.0	23.2	.....	....	....
Wheat germ	115.3	66.0	.....	.....	2.6	8.1	....

may be the η-tocopherol reported earlier (7) and identified as 7-methyltocol. No δ-T-3 was found in any of the samples examined.

Special attention has been given the possibility of finding evidence of the six unreported tocols and tocotrienols. A few samples have given peaks at the right retention times on all three phases for one or more or these compounds, but rechromatographing collected fractions failed to confirm these tentative identifications. They are present, if at all, at very low concentrations.

The method described here is a sensitive and specific tool for the analysis of the various tocopherol forms. For some samples it may be the method of choice, if not the only method available. But many sources, as indicated in Table VII, contain only a few easily separable forms that might also be analyzed on shorter GLC columns in less time than is required with those used here.

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