Determination of Tocopherols, Sterols, and Steryl Esters in Vegetable Oil Distillates and Residues

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ABSTRACT

A twofold technique for determination of tocopherols and a gas liquid chromatography procedure for determination of sterols and steryl esters are described. Incorporated herein are a modified Emmerie-Engel procedure for total tocopherols and gas liquid chromatographic analysis for tocopherols and sterols as their propionate esters. The approach is directly applicable to quality control and production use.

INTRODUCTION

The complexity of crude vegetable oil distillates and residues necessitates utilization of more than one technique for their accurate analysis. Interfering substances frequently are encountered which, by their reduction of iron (III) in the Emmerie-Engel (1) assay, are determined colorimetrically as tocopherols. Similarly, gas liquid chromatographic (GLC) analyses are plagued by interferers which coelute with tocopherols, sterols, or internal standards. The technique herein described, employing a colorimetric determination on the methanol soluble portion of the sample and GLC analysis of saponified and unsaponified portions of the sample, effectively reduces these interferences. A more accurate estimate of the actual tocopherol content is, thus, achieved.

EXPERIMENTAL PROCEDURES

Chemicals

Chemicals used were: potassium hydroxide (B&A reagent, American Chemical Society [ACS] Code 2118 [2]); 3A denatured alcohol (purified by distillation from potassium permanganate and potassium hydroxide); methanol (Eastman 13032); ethyl ether (absolute, MCB reagent ACS, must be peroxide free); pyridine (Eastman H214); propionic anhydride (Eastman 1291); ferric chloride (Baker analyzed reagent 1996); 2,2′-bipyridine (Eastman 4397); palmitoyl chloride (Eastman 2223); 1-hexadecanol (A305, Lachat Chemicals Inc., Chicago, Ill.); and *d*-α-tocopherol (Eastman 6340). Stigmasterol (Eastman 9128) also was used. It was purified in laboratory by successive crystallization from acetone. Sterol-4-ene-3-ones were used; they were laboratory prepared from soya sterols by the method of Kleiderer and Kornfeld (3) and purified on neutral alumina Brockman III. Sterol hydrocrabons, isolated from vegetable oil residues by chromatography on neutral alumina Brockman II, were used, as well as alpha-amyrin (20236) from K&K Laboratories, Plainview, N.Y. (This material contained an appreciable amount of β-amyrin.) Cetyl palmitate (hexadecanyl palmitate) was used. It was laboratory prepared from hexadecanol and palmitoyl chloride and purified by crystallization from 3A alcohol.

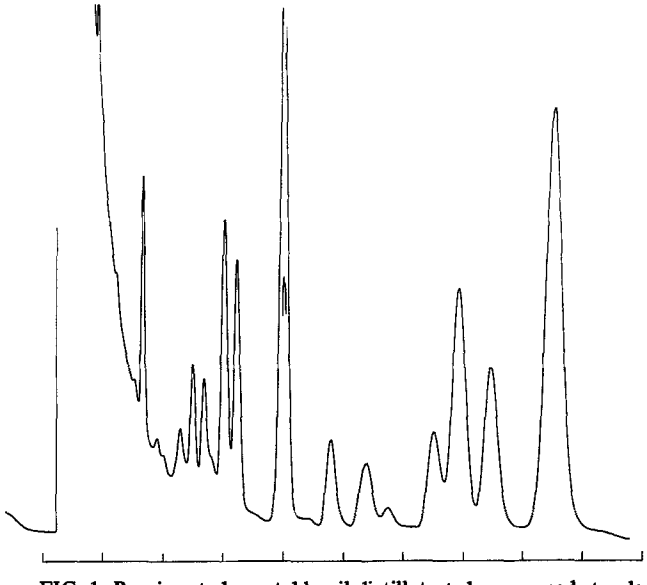

FIG. 1. Propionated vegetable oil distillate + decomposed sterols. Peak 1 = squalene; 2, 3, 4 = sterol hydrocarbons (3 peaks); 5 = δ tocopheryl propionate; 6 = β + γ tocopheryl propionate; 7 = α tocopheryl propionate; 8 = campest-4-ene-3-one; 9 = stigmast-4,22-diene-3-one; 10 = sitost-4-ene-3-one; 11 = campesteryl propionate; 12 = stigmasteryl propionate; and 13 = sitosteryl propionate.

GLC Column

Packing: To 90 ml toluene solution containing 0.90 g SE-52 (Supelco, Bellefonte, Pa.) were added 15.0 g of Chromosorb W-AW-DMCS (CRS Inc., Addison, Ill.). The mixture was allowed to stand at room temperature for 70 min with occasional gentle stirring, after which the packing was filtered to dryness on a Buchner funnel. A 244 cm x 0.4 cm inside diameter glass column was packed utilizing suction and gentle tapping.

Conditioning: With 0 helium flow and the column attached to the injection port only, the oven was heated from ambient to 280 C at 1 C/min and held at this temperature for 64 hr. The oven temperature then was lowered to 270 C and the helium flow adjusted to 70 ml/min. The column was ready for use after 2 hr under these conditions.

Apparatus

Analyses were performed on a Hewlett-Packard model 5750 gas chromatograph equipped with a flame ionization detector. Operating conditions were: injection port temperature, 280 C; column temperature, 255 C; detector temperature, 300 C; helium flow rate, 70 ml/min; hydrogen flow rate, 60 ml/min; air flow rate, 550 ml/min; electrometer input range 10^{-10} amp; and electrometer sensitivity 10^{-12} amp at 1 mv output. Peaks were measured by an Infotronics model CRS 101 electronic integrator.

Procedure

Ca. 50 g molten sample were weighed into a 250 ml round bottom flask and dried at reduced pressure on a steam bath. Concurrently, the water content of the sample was determined by azeotropic distillation from toluene.

Colorimetric method: Ca. 20 g (±0.01 g) of dried molten sample were weighed into a 250 ml Erlenmeyer flask. To this were added 150 ml absolute methyl alcohol, and the mixture was agitated thoroughly at 25 C for 5 min with a rapidly revolving glass stirrer. After the mixing was stopped, the mixture was allowed to stand 5 min after which the supernatant phase was decanted into a 500 ml volumetric flask. This procedure was repeated twice more combining

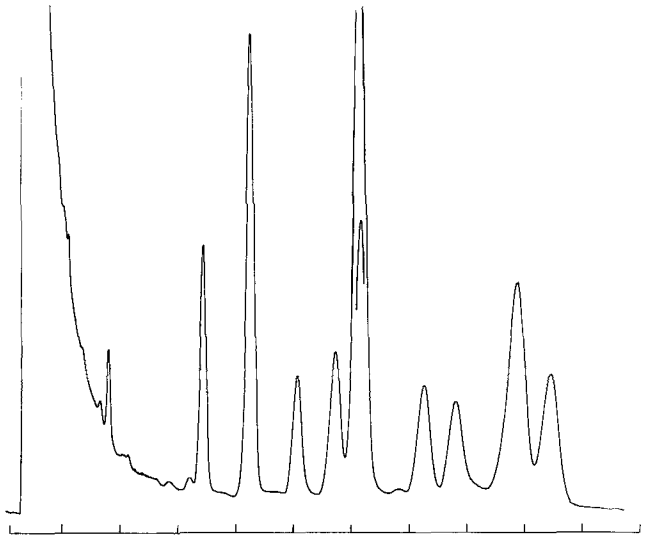

FIG. 2. Propionated vegetable oil distillate with added impurities. Peak 1 = squalene; 2 = δ tocopheryl propionate; 3 = β + γ tocopheryl propionate; 4 = α tocopheryl propionate; 5 = cholesteryl propionate; 6 = brassicasteryl propionate; 7 = campesteryl propionate; 8 = stigmasteryl propionate; 9 = sitosteryl propionate + β amyrin propionate; and 10 = α amyrin propionate.

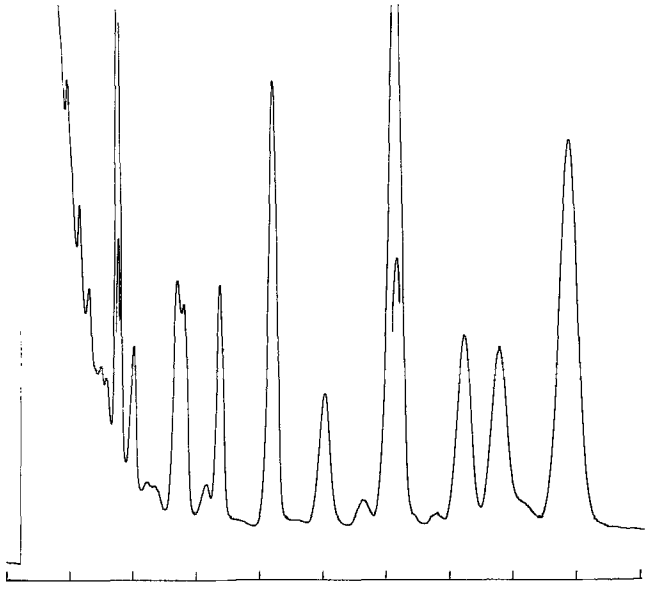

FIG. 3. Typical chromatogram of propionated vegetable oil distillate. Peak 1 = δ tocopheryl propionate; 2 = β + γ tocopheryl propionate; 3 = α tocopheryl propionate; 4 = cetyl palmitate; 5 = campesteryl propionate; 6 = stigmasteryl propionate; and 7 = sitosteryl propionate.

the extracts. The final volume was adjusted to 500 ml with absolute methyl alcohol; the flask thoroughly shaken; and the solution allowed to settle. An aliquot then was withdrawn for total tocopherol determination by the method of Rawlings (4).

GLC method: Into a 25 ml volumetric flask containing 50 mg (±0.01 mg) of well mixed dried oil was pipetted a chloroform solution containing cetyl palmitate (as an internal standard) equivalent in mg to the estimated tocopherol content of the weighed sample. The chloroform was removed under nitrogen on mild steam and 3 ml propionating reagent (2:1, propionic anhydride-pyridine) added. The sample then was reacted for 0.5 hr on a 75 C hot plate after which the reagents were removed under a stream of nitrogen on mild steam. The sample then was diluted with chloroform to ca. 1 mg/ml internal standard and 2-3 μliter injected on the column. A second wt was prepared without internal standard and analyzed to detect any interference in the internal standard region.

For saponification, 0.5 g (±0.1 mg) of dried oil was weighed into a 250 ml saponification flask and 50 ml 3A alcohol added. An air condenser was affixed and the solution refluxed on a hot plate for 5 min to dispel the air. While under reflux, 1 g potassium hydroxide pellets was added down the condenser and the sample allowed to react for 0.5 hr. While still under reflux, 4 ml 1:1 hydrochloric acid-distilled water were added down the condenser. The flask then was cooled under tap water and its contents transferred quantitatively to a 500 ml separatory funnel with 200 ml peroxide-free diethyl ether. The ether solution was washed 5 times with 75 ml portions of distilled water, transferred quantitatively to a 250 ml volumetric flask, and made to volume with peroxide-free ether. The flask then was shaken thoroughly and 25 ml pipetted into a 50 ml volumetric flask. A chloroform solution of internal standard was added and the sample prepared for analysis as before. Concurrently, a second 25 ml aliquot was analyzed without internal standard to determine possible interference in the internal standard region of the chromatogram.

Standardization routine: Each day, prior to sample analysis, the chromatographic system was standardized by means of a primary and secondary standard.

The primary standard solution was prepared in the following manner. Into a 25 ml volumetric flask were accurately weighed 25 mg pure *d*-α-tocopherol, 25 mg pure stigmasterol, and 25 mg internal standard. The primary standard was propionated as above and made to volume with chloroform after removal of reagents. Injections (2 μliters) of this solution were made until a constant wt response factor was obtained (generally 2 or 3 injections).

The secondary standard was a vegetable oil distillate of concentration similar to that of the oils being analyzed. Its purity was determined by replicate GLC analyses over a period of months. The analysis of this standard had to be within established limits of the assay prior to sample determination.

Calculations: The percentage of the individual tocopherols and sterols was determined from GLC data by means of the following equation:

$$\text{Percent} = \frac{A_S}{W_S} \times \frac{W_{IS}}{A_{IS}} \times F \times 100 \times H$$

where A_S = area of the tocopheryl or steryl propionate peak; W_S = wt of sample in mg; W_{IS} = wt in mg of internal standard added to the sample; A_{IS} = area of internal standard peak corrected for interfering peaks; F is the relative wt response factor calculated from the primary standard by means of the following equation:

$$F = \frac{A_{IS}}{W_{IS}} \times \frac{W_S}{A_S}$$

and H is the correction for water in the sample:

$$H = 1 - \frac{\%\,H_2O}{100}$$

The percentage of steryl esters was determined by comparison of saponified and unsaponified portions of sample as:

$$\text{Percent} = (S - U)E$$

where S = percent sterols determined on the saponified sample; U = percent sterols determined on the unsaponified

TABLE I

Retention Times of Pertinent Compounds Relative to Cetyl Palmitate

Compound	Relative retention time
Cetyl palmitate (t_r = 26-27 min)	1.00
Squalene	0.27
C-16 Glyceryl monoester propionate	0.28
C-18 Glyceryl monoester propionate	0.43-0.46
Sterol hydrocarbons (3 peaks)	0.41-0.49
δ-Tocopheryl propionate	0.54
β- and γ-Tocopheryl propionate	0.68
α-Tocopheryl propionate	0.81
Campest-4-ene-3-one	0.90
Cholesteryl propionate	0.91
Stigmast-4,22-diene-3-one	0.97
Brassicasteryl propionate	1.04
Sitost-4-ene-3-one	1.13
Campesteryl propionate	1.17
Stigmasteryl propionate	1.27
β-Amyrin propionate	1.41
Sitosteryl propionate	1.45

sample; E = sterol to steryl ester conversion factor:

$$E = \frac{\text{Average mw of steryl esters}}{\text{Average mw of sterols}}$$

In the colorimetric assay, since the tocopherol homologue ratio may vary greatly between oils and since β, γ, and δ tocopherols react to give larger values stoichiometrically than would be expected (5,6), it was necessary to correct the Emmerie-Engel value by a factor based upon GLC data. The factor was determined by means of the following equation:

$$F_E = \frac{1000}{1000A \times 1116G \times 1345D}$$

where 1000, 1116, 1345 = the relative Emmerie-Engel responses (mg/g) for pure α, γ, and δ tocopherols, respectively (pure β tocopherol = 1086 mg/g but does not usually occur in significant amounts in crude distillates) and A, G, D = the ratio percents of each homologue in a given sample.

DISCUSSION

Extraneous peaks may appear in varying profusion on the chromatograms of vegetable oil distillates and residues. Sterol hydrocarbons and sterol ketones (Fig. 1) generally result from dehydration and oxidation during processing while the frequent, naturally occurring interferers are cholesterol, brassicasterol, β-amyrin, and various glyceride esters (Fig. 2).

TABLE III

Comparison of Total Tocopherols[a] in Vegetable Oil Distillates and Residues by Emmerie-Engel, Methanol Extraction, and Gas Chromatography

Sample	Emmerie-Engel	Corrected[b] Emmerie-Engel	Methanol extraction	Gas liquid chromatography
1	118	103	91	89.5
2	125	109	96	97.6
3	80	69	64	64.6
4	105	92	84	83.2
5	75	65	55	52.4
6	99	86	83	79.7
7	89	78	70	68.6
8	78	68	67	63.8
9	98	86	79	78.5
10	97	83	75	75.3
11	89	78	66	68.5
12	74	64	54	52.0
13	76	66	61	59.6
14	70	61	55	55.0
15	84	73	70	68.5

[a] All values are mg/g.

[b] Emmerie-Engel values were corrected by the same factor which was utilized in the methanol extraction technique.

Several liquid phases (SE-52, SE-30, OV-101, OV-3, OV-17, and SP-400) and several derivatives (trimethylsilyl ethers, acetates, propionates, and butyrates) have been investigated. Propionate derivatives chromatographed on SE-52 or SP-400 (as indicated by the McReynolds constants, GLC columns prepared of SP-400 chlorophenyl silicone were identical to SE-52) effected the best separation of tocopherols and sterols from interferers (Table I). Briefly, other combinations suffered the following drawbacks: trimethylsilyl ether derivatives of γ, β, and δ tocopherols coeluted with sterol hydrocarbons; sterol trimethylsilyl ethers coeluted with sterol ketones; δ tocopheryl acetate coeluted with the hydrocarbons; steryl acetates coeluted with ketones; and butyrate derivatives of the tocopherols were resolved from the hydrocarbons but coeluted with ketones. Significant interferers which coeluted with the cetyl palmitate on SE-52 and SP-400 were brassicasterol and stigmast-4,22-diene-3-one.

β-Amyrin, present in some of the oils investigated, coeluted with β-sitosteryl propionate on SE-52 but was resolved completely on an OV-225 column (relative retention vs. β-sitosteryl propionate = 1.1) prepared in the manner described for SE-52.

The column conditioning procedure includes an extended period of no-flow conditioning at an elevated temperature. This was found to be necessary to eliminate

TABLE II

Comparison of Tocopherol Analyses[a] by Gas Liquid Chromatography on Unsaponified and Saponified Samples

	Unsaponified				Saponified			
Sample	α	β-γ	δ	Total	α	β-γ	δ	Total
1	6.8	58.8	20.6	86.2	6.8	60.0	21.3	88.1
2	7.3	49.8	18.5	75.6	7.3	49.0	18.7	75.0
3	10.9	25.8	9.5	46.2	6.9	26.1	10.1	43.1
4	16.8	37.6	14.6	69.0	9.2	38.5	14.0	61.7
5	5.7	51.7	24.5	81.9	5.7	52.8	26.1	84.6
6	10.1	106.3	52.3	168.7	10.0	107.0	52.3	169.3
7	9.3	21.7	8.2	39.2	8.7	22.1	8.6	39.4
8	10.2	73.0	31.0	114.2	10.7	74.5	31.9	117.1
9	9.2	87.6	43.2	140.0	9.1	88.0	44.0	141.1
10	8.9	29.9	12.2	51.0	6.1	30.2	12.4	48.7
11	11.1	25.7	9.5	46.3	7.2	27.0	9.7	43.9

[a] All values are mg/g.

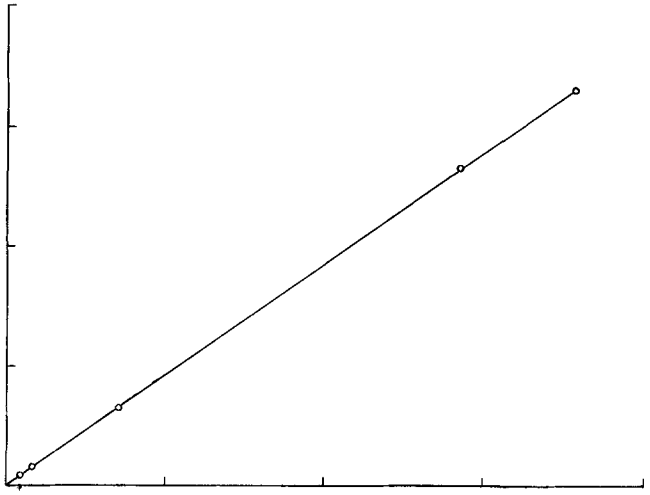

FIG. 4. Relative wt response of *d*-α-tocopheryl propionate-cetyl palmitate. Less than 5 μg of each component injected.

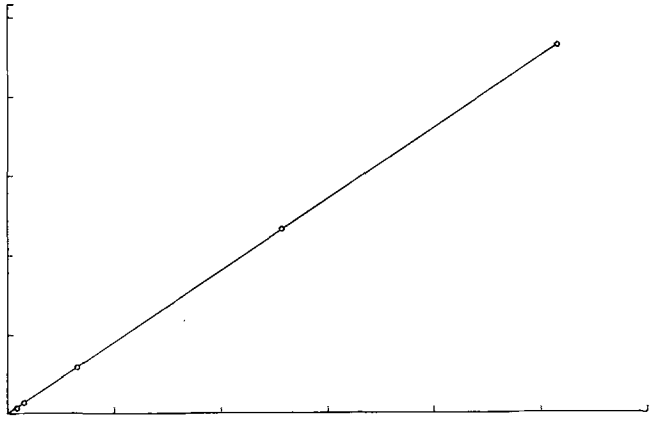

FIG. 5. Relative wt response of stigmasteryl propionate-cetyl palmitate. Less than 5 μg of each component injected.

column adsorption which was observed, via dose-response calibration, when shorter periods of conditioning were employed.

Chromatographic columns prepared in the manner described exhibited efficiencies of 3200-4000 plates and a stigmasteryl propionate to campesteryl propionate resolution factor of 1.10-1.30 (Fig. 3). Some loss of efficiency was observed after 3-4 weeks of constant use at which time the columns were replaced. The responses of *d*-α-tocopheryl propionate and stigmasteryl propionate relative to cetyl palmitate were determined over the range of wt ratios normally encountered (Figs. 4 and 5) and the equations of the lines calculated by the method of least squares. The lines passed practically through the origin (intercepts of -0.029 and 0.004, respectively) and had standard deviations of 0.003 and 0.003, respectively. Column overloading was observed when injections exceeded 5 μg of any component.

Following saponification, an acidulation step was incorporated. This technique, not only prevented caustic oxidation of the sample, but also eliminated emulsions which often hindered the wash up when other techniques were employed (7).

Correction for peaks eluting at the time of the internal standard was made in the manner discussed below.

For the injection without internal standard, a ratio was obtained between the interfering peak and the γ-tocopheryl propionate peak; this factor was applied to the γ-tocopheryl propionate peak on the corresponding chromatogram with internal standard. The resulting, calculated, interference area was subtracted from the apparent internal standard area.

Tocopherol potency was determined on both saponified and unsaponified portions of sample, thus providing a check on this determination. Occasionally, glyceryl ester interference with α-tocopheryl propionate was encountered, in which case the lower saponified value was taken. The results of several analyses chosen at random are listed in Table II.

The sterol-to-steryl ester conversion factor (E) was calculated from GLC data for each of several different vegetable oils using the formula:

$$E = \frac{(M_S + M_A)\text{-}18}{M_S}$$

where M_S = average mol wt of the sterols determined on the saponified sample, and M_A = average mol wt of the fatty acids in the sample (8). The values ranged from 1.61-1.63 with an overall average of 1.62. As a routine, this value was verified every 6 months.

Colorimetric (Emmerie-Engel) Method (1)

The colorimetric (Emmerie-Engel) method sometimes employed to determine the vitamin E content of vegetable oil distillates and residues is not specific, since any compound which is oxidized by the Emmerie-Engel reagent will be determined as vitamin E. As a result, values by this method, even after correction for homologue ratio, are frequently high. The methanol extraction modification procedure has been utilized as an internal laboratory control technique to supply values which: (A) if exceeded by the GLC assay, indicated possible coelution of interferers with the tocopherols or (B) if the GLC assay was too low by comparison, confirmation was made by reassay.

A comparison of total tocopherols by: (A) Emmerie-Engel, (B) Emmerie-Engel values corrected for homologue response, (C) methanol extraction, and (D) GLC can be found in Table III.

To obtain a rough estimate of tocopherol content by the methanol extraction technique without the aid of GLC data, the following approximate correction factors may be used on distillates from the following vegetable oils: soya, 0.86; corn, 0.92; cottonseed, 0.94; peanut, 0.94; sunflower, 0.95; and safflower, 0.93.

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