Gas Chromatographic Determination of Tocopherols and Sterols in Soya Sludges and Residues — an Improved Method

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Abstract

An improved method is described for determining the tocopherol and sterol content of soya sludges and residues. Samples are saponified and the extracted unsaponifiables are derivatized prior to gas chromatography to eliminate interferences. Data are presented to demonstrate the improvements realized.

Introduction

A previous publication from our laboratory described a gas chromatographic analysis for tocopherols and sterols in soya sludges and residues (1). Briefly, the analysis involved saponification of the sample followed by extraction and gas chromatography of the unsaponifiables. Several significant modifications have been made on this method with resulting improvement in precision and accuracy. The major modification is conversion of the tocopherols and sterols to butyrate esters prior to gas chromatography. This eliminates interference from materials encountered commonly in the sludges and residues.

Experimental Procedures

Reagents and Apparatus

Potassium Hydroxide Solution. Potassium hydroxide, 160 g, was dissolved in 100 ml of distilled water.

Pyrogallol Solution. Pyrogallol, 5 g, was dissolved in 95 ml of absolute ethyl alcohol. This solution was prepared fresh each day. The bottle of pyrogallol, used to prepare the solution, was replaced every six months.

α-Tocopherol Standard. α-Tocopherol was purchased from Distillation Products, Industries, Rochester, N.Y.

Stigmasterol Standard. Stigmasterol from soya sludge was purified by liquid column chromatography on Florisil. The purified product had a melting point of 169.5–170.5 C.

Cholesterol. Cholesterol was purchased from Merck, Inc., Rahway, N.J.

Isovaleric Anhydride. Isovaleric anhydride was purchased from K and K Lab., Plainview, N.J.

Cholesteryl Isovalerate. Cholesterol, 100 g, was placed in a 1 liter round bottom flask and a solution containing 100 g isovaleric anhydride in 200 ml pyridine was added. After refluxing 2 hr the solution was cooled and poured into 500 ml of cold water maintained at 5 C. After 1 hr, the crystals were filtered and washed with 200 ml of cold (5 C) acetone. This material was recrystallized from acetone and dried overnight in a 70 C vacuum oven.

Cholesteryl Isovalerate-Butyric Anhydride Solution. About 0.15 g cholesteryl isovalerate was accurately (±0.1 mg) weighed into a 50 ml volumetric flask, dissolved and diluted to the mark with a solution consisting of 2 parts pyridine and 1 part butyric anhydride. The cholesteryl isovalerate solution must be prepared fresh every three days.

Butyric Anhydride. Butyric anhydride was purchased from Distillation Products Industries, Rochester, N.Y. The butyric anhydride was distilled using an unpacked Vigreux column (8 in. long). After discarding a 10% forecut, a 70% to 80% heart cut was collected.

Gas Chromatograph Column Packing. To 10 g of Gas Chrom Q (Applied Science Lab., State College, Pa.) in a 500 ml flask was added 100 ml of a toluene solution containing 0.1 g silicone gum rubber SE-30 (General Electric Co., Schenectady, N.Y.). The solvent was stripped with a water aspirator while the mixture was stirred and heated in a water bath at 70 C. After the packing became free-flowing, it was transferred to a Petri dish and heated at 70 C on a hot plate until no solvent odor was detected.

Gas Chromatographic Column. The column was prepared as described previously (1).

Gas Chromatography. Analyses were performed on a Model 400 flame ionization gas chromatograph manufactured by the Hewlett Packard Co., Avondale, Pa. The operating conditions of the chromatograph were: column temperature 235 C, injection port temperature 275 C, detector temperature 250 C, range 100, attenuation 4, hydrogen flow rate 60 cc/min, helium flow rate 170 cc/min, air flow rate 300 cc/min.

Procedure

Accurately (±0.1 mg) weigh about 5 g of molten sample into a 500 ml volumetric flask. Dissolve and dilute to the mark with benzene. Pipet a 25 ml aliquot into a 50 ml round bottom flask and evaporate the solvent on a steam bath under a stream of nitrogen (requires about ½ hour). Then add 4 ml of pyrogallol solution to the residue and attach a water-cooled condenser to the flask. After heating the solution to reflux, add 1 ml of potassium hydroxide solution and continue refluxing an additional 3 min. Remove the flask and cool under tap water.

Add 20 ml distilled water to the flask and transfer the solution quantitatively to a 125 ml separatory funnel. Use two 10 ml portions of diethyl ether to rinse the flask for quantitative transfer. Extract the solution in the separatory funnel with the diethyl ether, taking care not to shake too vigorously since emulsions tend to form. Extract the solution five more times with 25 ml portions of diethyl ether and combine the extracts. Transfer the combined extracts to a separatory funnel and wash with 20 ml portions of distilled water until the washings are neutral to phenolphthalein indicator (usually requires four to eight washings).

Concentrate the diethyl ether solution to 25 ml under nitrogen on a steam bath, then transfer quantitatively to a 50 ml round bottom flask. Evaporate the diethyl ether under nitrogen on a steam bath. Then add 10 ml of 5 to 1 benzene-ethyl alcohol solution and also evaporate this solvent under nitrogen on a steam bath. If traces of water are still visible in the flask, repeat the addition and evaporation of benzene-ethyl alcohol solution.

Pipet 8 ml of cholesteryl isovalerate-butyric anhydride solution into the residue in the flask. Reflux the solution 10 min. and then cool under tap water. Inject approximately 2.5 μl of this solution into the gas chromatograph.

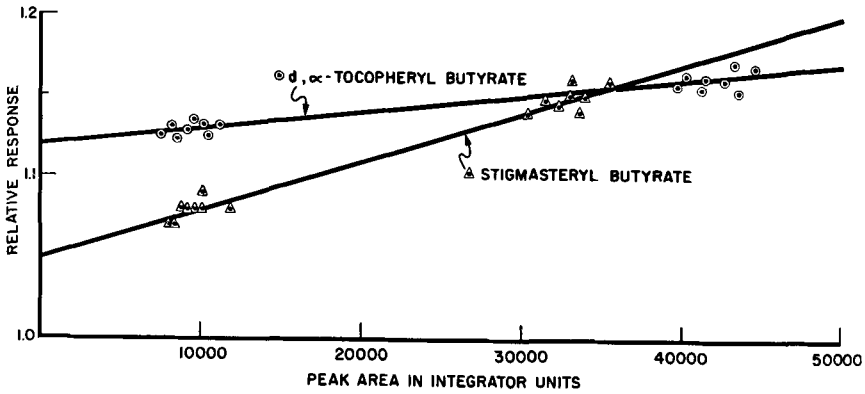

FIG. 1. Tocopheryl and steryl butyrate relative response vs. peak area.

Calculations

The areas of the tocopheryl butyrate, steryl butyrate and cholesteryl isovalerate peaks were measured by electronic integration. The percent of the individual tocopherols or sterols was calculated by means of the following equation:

$$\text{Per cent tocopherol or sterol} = \frac{A \times W \times 100 \times 20}{RR \times A_s \times S}$$

where A, area of tocopheryl or steryl butyrate peak; A_s, area of cholesteryl isovalerate peak; W, milligram of cholesteryl isovalerate added to the sample; S, original sample weight in milligrams; and RR, relative response of tocopheryl or steryl butyrate (obtained from Fig. 1).

Calibration

Purified α-tocopherol and stigmasterol were used as the standards for determining the relative response values for tocopherols and sterols, respectively. Calibrations were performed in the following manner. Known mixtures of pure α-tocopherol and pure stigmasterol were prepared to have the following composition. (a) Mixture 1: 50 mg, α-tocopherol; 70 mg, stigmasterol; 50 ml, cholesteryl isovalerate-butyric anhydride. (b) Mixture 2: 130 mg, α-tocopherol; 80 mg, stigmasterol; 10 ml, cholesteryl isovalerate butyric anhydride.

The above solutions were refluxed 10 min, cooled and chromatographed in duplicate. The preparation and gas chromatography of the mixtures was repeated on three other days. Peak areas were measured by electronic integration and relative response values were calculated by means of the following equation.

$$\text{Relative response (RR)} = \frac{A \times W_s}{W \times A_s}$$

where A, area of stigmasteryl butyrate or α-tocopheryl butyrate; A_s, area of cholesteryl isovalerate; W, milligram of stigmasterol or α-tocopherol; and W_s, milligram of cholesteryl isovalerate.

Peak areas of α-tocopheryl butyrate and stigmasteryl butyrate were plotted vs. relative response as shown in Figure 1.

TABLE I

Relative Retention Times of Tocopheryl and Steryl Butyrates

Compound	Relative retention time[a]
δ-Tocopheryl butyrate	0.46
β + γ-Tocopheryl butyrate	0.61
α-Tocopheryl butyrate	0.75
Campesteryl butyrate	1.12
Stigmasteryl butyrate	1.23
β-Sitosteryl butyrate	1.40

[a] Relative to cholesteryl isovalerate.

Discussion

The old method did not resolve completely the tocopherol peaks from small extraneous peaks during gas chromatography. In addition, recent studies showed that small interfering peaks, equivalent to approximately 1% (absolute) of tocopherol were hidden under the δ- and γ-tocopherol peaks. In the new method, the tocopheryl butyrate peaks are displaced away from all interfering and unresolved peaks with a resultant improvement in accuracy. In contrast, derivatization of the tocopherols to the acetates, propionates and the dimethyl- or trimethylsilyl ethers did not succeed in eliminating these interferences.

The relative retention times for the butyrate esters of the tocopherols and the sterols in soya sludges and residues are listed in Table I. These data were obtained from gas chromatograms of samples of known composition. The butyrate esters of β and γ tocopherols are not resolved from each other and are determined together as β plus γ tocopherol. During analysis, peaks are identified by comparing peak relative retention times with those in Table I. Figure 2 is a

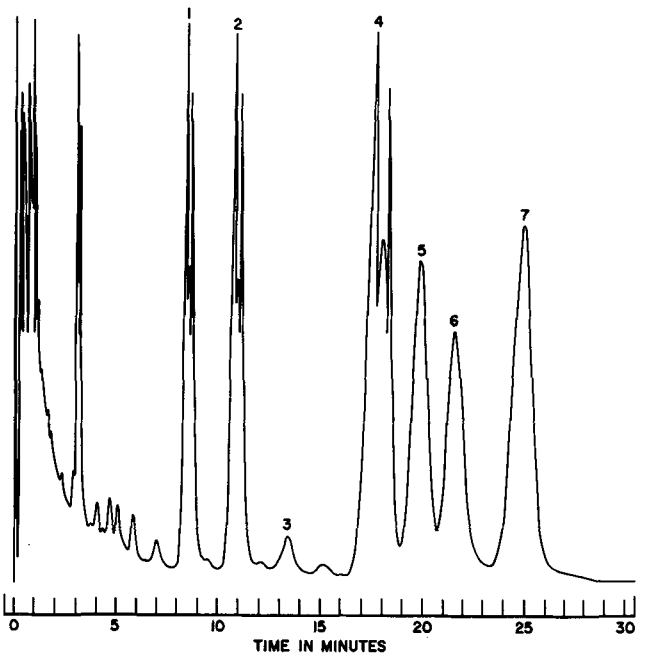

FIG. 2. Gas chromatogram of soya sludge unsaponifiables. Peak identification: 1, δ-tocopheryl butyrate; 2, β + γ-tocopheryl butyrate; 3, α-tocopheryl butyrate; 4, cholesteryl isovalerate; 5, campesteryl butyrate; 6, stigmasteryl butyrate; 7, β-sitosteryl butyrate.

TABLE II

Precision, Old vs. New Method

	Coefficient of variation	
	Old method (%)	New method (%)
Total tocopherol in a soya sludge[a]	3.3	1.0
Total sterol in a soya sludge[a]	1.8	1.4
Total tocopherol in a tocopherol concentrate from soya sludge[b]	3.0	0.8
Total sterol in a sterol concentrate from soya sludge[c]	2.5	0.9

[a] Based on 16 analyses by each method over a period of two months.
[b] Based on 50 analyses over a one-year period.
[c] Based on 40 analyses over a one-year period.

typical gas chromatogram obtained during the analysis of a soya sludge.

The improved precision of the new method is shown in Table II. Relative standard deviations of the two methods on the same sludge sample are compared. The new method is more precise for several reasons. One reason is the butyrylation, as evidenced by the data in the same table where results on tocopherol and sterol concentrates with and without butyrylation are compared.

Another reason for the improved precision of the new method is the increased number of extractions employed. Other workers (2) had recommended three extractions for similar materials but, as shown in Table III three extractions of the saponification mixture did not recover all the sterol and tocopherol.

Improvements in precision and accuracy also have resulted from several other procedural changes incorporated in the new method. The use of large samples for analysis in the new method minimizes nonrepresentative sampling which is a concern with the

TABLE III

Effect of Increasing Number of Extractions

Number of extractions	Total tocopherol,[a] %	Total sterol,[a] %
3	9.5	13.2
6	10.4	14.2
9	10.4	14.3

[a] Averages of duplicate analyses.

often heterogeneous soya sludges. Also, the azeotropic distillation of water from the extracted unsaponifiables ensures complete butyrylation of the tocopherols and sterols. Experience has shown that grossly incomplete butyrylation of sterols results if substantial water is present during butyrylation.

For accurate and precise results the gas chromatographic column must perform properly. Two criteria are recommended for determining proper performance, theoretical plate number and peak resolution. The theoretical plate number for the stigmasterol peak should be determined and only columns with higher than 2000 theoretical plates should be used.

The separation of the campesteryl butyrate and stigmasteryl butyrate peaks was chosen as a measure of peak resolution because campesterol and stigmasterol are present in approximately equal amounts in soya sludges and residues. Peak resolution of at least 1.07 is recommended. Values for columns which have performed well in this laboratory have ranged from 1.07 to 1.14.

REFERENCES

1. Nelson, J. P., and A. J. Milun, JAOCS *45*, 848–851 (1968).
2. Analyst *84*, 361–362 (1959).

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