Gas Chromatographic Determination of Tocopherols and Sterols in Soya Sludges and Residues

J. P. NELSON and A. J. MILUN, General Mills, Inc., 2010 East Hennepin Avenue, Minneapolis, Minnesota 55413

Abstract

A method is described for determining the individual and total sterols and tocopherols of soya sludges and residues. The method involves saponification of the sample followed by gas chromatographic analysis of the unsaponifiables. Data are presented on the reproducibility and accuracy of the method.

Introduction

SOYBEAN OIL is one of the sources of naturally occurring tocopherols and sterols. In the deodorization of this oil, a concentrate of the tocopherols and sterols is obtained which is called deodorizer distillate. A second type of concentrate is obtained when after hydrolysis of soybean acid soapstock, the fatty acids are distilled. The residue from the distillation is a good source of sterols.

In the past, it has been common to employ two separate analytical methods to determine the tocopherol and sterol content of soya sludges and residues. Total tocopherol was determined by the Emmerie-Engel method (5) and total sterol by the digitonide procedure (7). Neither of these methods is specific, that is, any compound which is oxidized by the reagent in the Emmerie-Engel method will be counted as tocopherol, and with the digitonide procedure for sterols, any compound that will precipitate with digitonin is counted as a sterol. Furthermore, these methods do not differentiate among the different types of sterols and tocopherols that usually are present.

In recent years publications have appeared describing the gas chromatography of sterols and tocopherols (2–4,6,8–11); however none of these apply specifically to the by-products from processing of soybean oil. This paper presents a gas chromatographic procedure for determining the individual, as well as the total tocopherols and sterols in soybean oil deodorizer distillate and distillation residues. The procedure involves the saponification of samples to free the tocopherols and sterols that are present as esters. The tocopherols and sterols then are extracted with diethyl ether and the extracts subsequently gas chromatographed.

Experimental Procedures

Reagents and Apparatus

Squalane Internal Standard Solution. Approximately 0.1 g (±0.0001 g) of distilled squalane (Applied Science Laboratories, State College, Pa.) was weighed into a 100 ml volumetric flask and diluted to the mark with reagent grade chloroform.

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.

α-Tocopherol Standard. This compound was purchased from Distillation Products Ind., Rochester, N.Y.

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

Gas Chromatograph Column Packing. Three hundred milliliters of a toluene solution containing 0.9 g of silicone gum rubber SE-30 (General Electric Co., Schenectady, N.Y.) was added to 26 g Gas Chrom Q (Applied Science Lab., State College, Pa.) in a one liter flask. The solvent was stripped off 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 glass column was 6 ft long with an internal diameter of 3.5 mm and bent in the shape of a U. Prior to packing, the column was rinsed with potassium dichromate-sulfuric acid cleaning solution for 5 min. Then 500 ml of distilled water were flushed through the column, followed by 200 ml of absolute methyl alcohol. Then the column was rinsed with 1% dimethyl dichlorosilane in chloroform, washed with 100 ml absolute methyl alcohol and dried by aspirating with air. With the aid of a vibrator the column was filled by pouring approximately 6 in. of packing alternately into each leg until the packing level was 2 in. from the inlet end and ½ in. from the outlet end. Finally, the ends of the column were plugged with glass wool. The glass wool had been rinsed previously with a 1% solution of dimethyl dichlorosilane in chloroform, washed with absolute methyl alcohol, and air dried.

The packed column was conditioned in the instrument first by flushing with helium for 5 min then, after turning off the helium flow, by heating at 245 C for 1 hr. At this time the helium flow was turned on and the instrument left at this temperature for 15 hr.

Gas Chromatograph. Analyses were performed on a Model 400 flame ionization gas chromatograph manufactured by Hewlett Packard, Avondale, Pa. The operating conditions of the chromatograph were: column temp. 225 C, injection port temperature 270 C, detector temperature 250 C, range 100, attenuation 16, hydrogen flow rate 50 cc/min, helium flow rate 150 cc/min, air flow rate 350 cc/min.

Approximately 0.2 g of sample was accurately (±0.0001 g) weighed into a 50 ml round bottom flask. Then, 4 ml of a 5% solution of pyrogallol in absolute ethyl alcohol were added to prevent oxidation of the tocopherols during saponification. A water cooled condenser was attached to the flask and the solution was heated to reflux, at which time 1 ml of 60% aqueous potassium hydroxide was added. The solution was allowed to reflux 3 min and the flask was removed and cooled under tap water.

Distilled water, 20 ml, was added to the flask and the solution was transferred quantitatively to a 125 ml separatory funnel. Two 10 ml portions of diethyl ether were used to rinse the flask for quantitative transfer. The solution in the separatory funnel was extracted with the diethyl ether, taking care not to

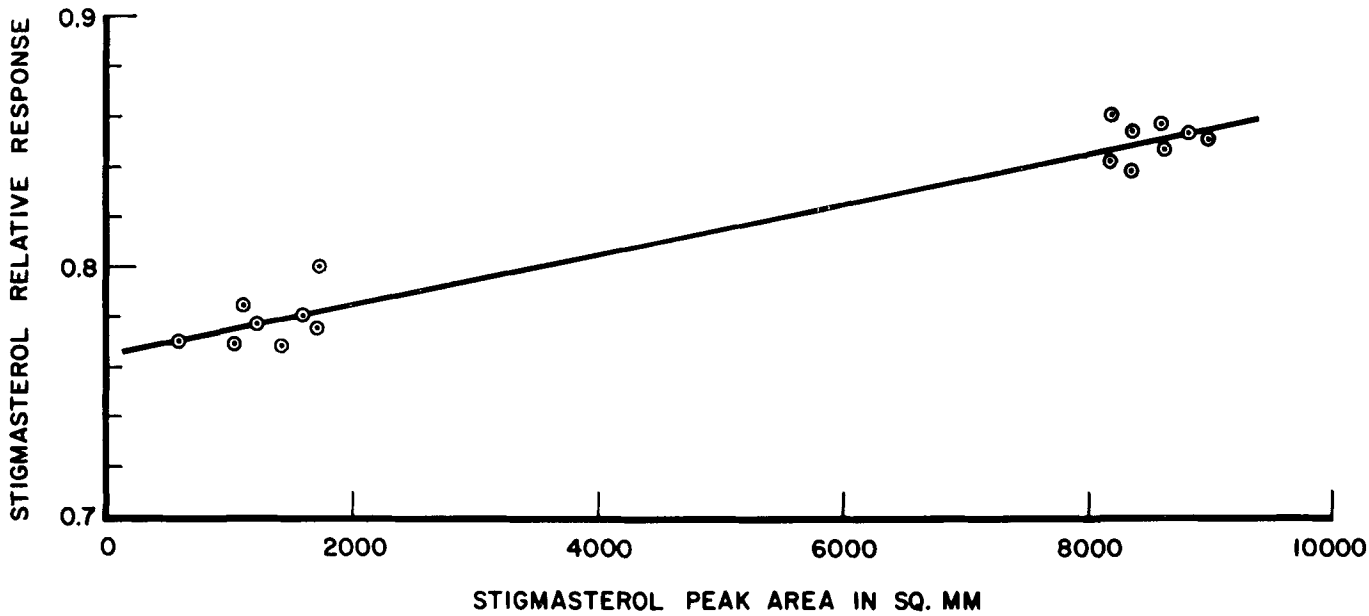

FIG. 1. Sterol relative response vs. sterol peak area.

shake too vigorously since emulsions tended to form. The solution was extracted two more times with 25 ml portions of diethyl ether and the three diethyl ether extracts were combined. The combined extracts were transferred to a separatory funnel and washed with 20 ml portions of distilled water until washings were neutral to phenolphthalein indicator (usually 4 to 8 washings were required).

The diethyl ether solution was then transferred to a 100 ml round bottom flask and the diethyl ether was evaporated under nitrogen on a steam bath. When the diethyl ether was no longer visible, 4 ml of squalane solution were added with a pipet and the flask was swirled to insure complete solution of the residue. Approximately 2.5 μl of this solution were injected into the gas chromatograph which had been previously adjusted to a chart speed of 40 in./hr and an attenuation of 32. Approximately 1 min after the apex of the squalane peak, the chart speed was changed to 20 in./hr and the attenuation to 16.

Calculations

The areas of the squalane, tocopherol and sterol peaks were calculated by means of triangulation, that is, peak height times peak width at ½ height. The per cent of the individual tocopherols or sterols were calculated by means of the following equation:

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

where: A = area of tocopherol or sterol peak; As = area of squalane peak; W = mg squalane added to the sample; S = sample weight in mg; and RR = relative response of tocopherol (average value found was 0.87) or sterol (obtained from Figure 1).

TABLE I
Relative Retention Times of Tocopherols and Sterols

Compound	Relative Retention Time[a]
Squalane	1.00
α-Tocopherol	3.40
β-Tocopherol	2.72
Δ-Tocopherol	2.05
γ-Tocopherol	2.76
Campesterol	3.90
Stigmasterol	4.32
Sitosterol	5.10

[a] Relative to squalane.

Calibration

α-Tocopherol and stigmasterol were used as the standards for determining the response values (RR) for tocopherols and sterols, respectively. Calibrations were performed in the following manner.

Known mixtures of α-tocopherol, stigmasterol and squalane were prepared to have the following composition:

Mixture	α-Tocopherol mg	Stigmasterol mg	Squalane Solution ml
1	40	120	50
2	80	80	10

These two known mixtures were prepared on four different days and gas chromatographed in duplicate each day. Peak areas were measured as described in the procedure 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 stigmasterol or α-tocopherol; A_s = area of squalane; W = mg of stigmasterol or α-tocopherol; and W_s = mg of squalane.

The average relative response for α-tocopherol was 0.87 with a standard deviation of $\pm$0.02 and did not change with concentration.

In contrast, the relative response for stigmasterol did vary with the amount of sample injected. Therefore the curve shown in Figure 1, a plot of peak area vs. relative response for stigmasterol, was drawn and used to determine the relative response, depending on the peak area of stigmasterol in a given sample. This variation of stigmasterol relative response was probably caused by column adsorption of the highly polar compound.

Results

The relative retention times determined for the tocopherols and sterols that are present in soya sludges and residues are listed in Table I. Identifications of peaks in samples of soya sludges and residues

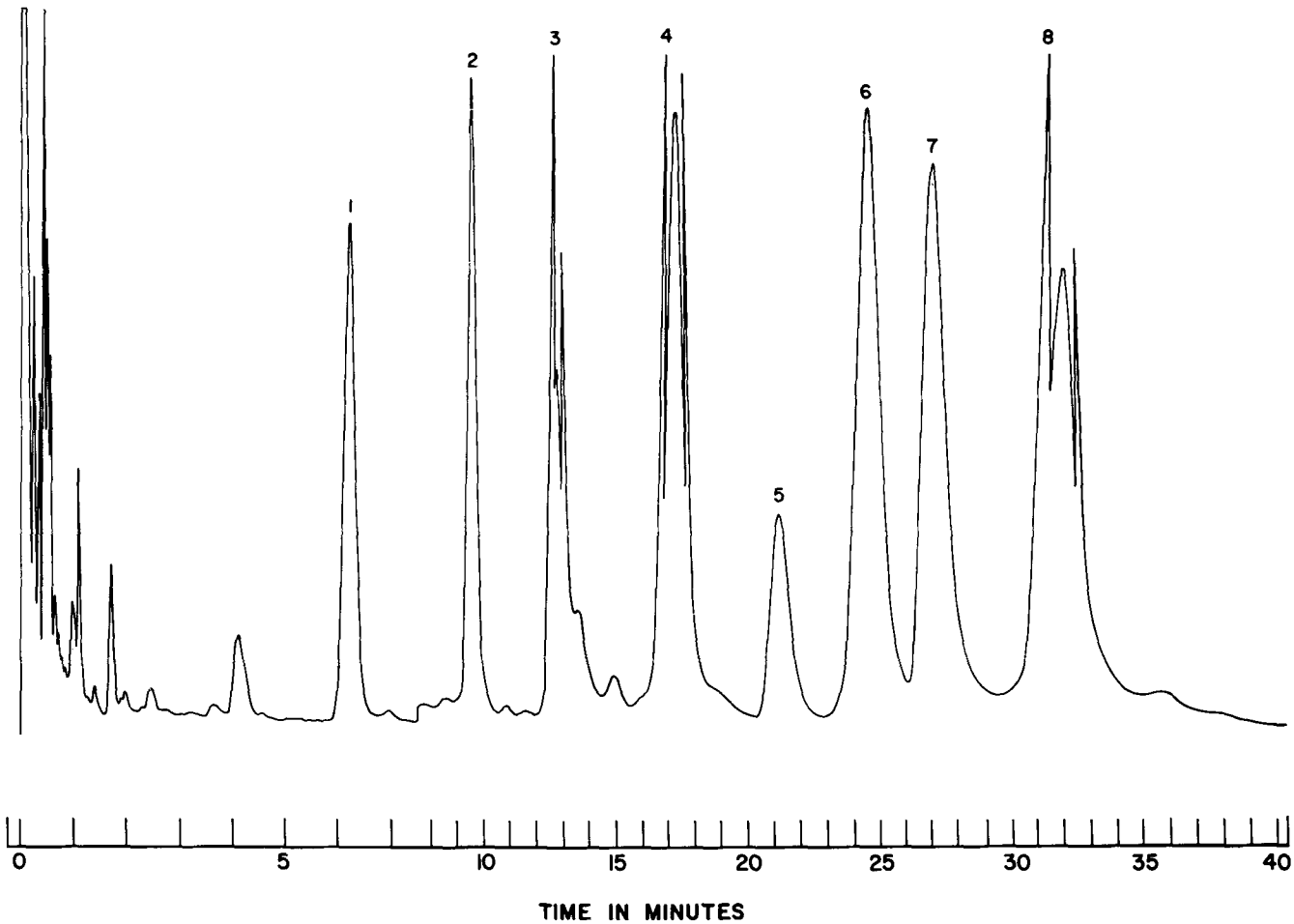

FIG. 2. Gas chromatogram of soya sludge unsaponifiables. Peak identification: 1. squalane, 2. squalene, 3. Δ tocopherol, 4. β + γ tocopherol, 5. α-tocopherol, 6. campesterol, 7. stigmasterol, 8. sitosterol.

were made by comparing peak relative retention times with those in Table I. The β- and γ-tocopherols are not resolved and therefore are determined together as β + γ tocopherol. Identifications were confirmed by trapping during elution from the gas chromatograph and subsequent infrared and mass spectral analysis. Figure 2 is a typical gas chromatogram of the unsaponifiables from a soya sludge.

Table II lists the results obtained on a reproducibility study of the method. A sample of soya sludge was analyzed 11 times over a three-week period and a soya residue sample was analyzed five times on as many days. The average percentages and standard deviations are listed.

Since only stigmasterol and α-tocopherol were available in high purity, these were used for quantitative calibrations. All sterols and tocopherols determined by this method were assumed to have the same relative responses. This is a reasonably valid assumption since there are only small differences in structure among the sterols and among the tocopherols. Two impurities, γ-tocopherol and an unidentified component, were detected in the α-tocopherol standard. These totaled 1.5% of the peak areas on the gas chromatogram. No impurities were detected in the stigmasterol standard.

The saponification procedure used is the one recommended in a report by the Vitamin E panel of the Analytical Methods Committee (1). To check this saponification, measured quantities of α-tocopherol, stigmasterol and sitosterol were added to a soya sludge which had been analyzed previously by the method described in this paper. These mixtures were saponified and re-analyzed. The results in Table III indicate that satisfactory recovery was obtained.

To check whether samples were completely saponified after refluxing 3 min in alcoholic potassium hydroxide, three different samples of soya sludge also were refluxed 20 min and analyzed. Comparison of results showed that a 3 min reflux was sufficient to give complete saponification. Actually, the results on the 20 min refluxed samples were 0.1% to 0.2% lower in total tocopherol content which might be an indication that some tocopherol was lost through degradation or oxidation. No effect on sterol content was noted with the longer reflux time.

TABLE II
Reproducibility of the Method

Sample	Tocopherols				Sterols			
	Δ %	β + γ %	α %	Total %	Camp. %	Stig. %	Sit. %	Total %
Soya sludge	3.0 ± 0.2	7.7 ± 0.3	1.5 ± 0.0	12.3 ± 0.4	5.7 ± 0.2	5.2 ± 0.2	11.0 ± 0.2	21.9 ± 0.4
Soya residue	0	0	0	0	7.5 ± 0.1	6.4 ± 0.1	15.5 ± 0.4	29.8 ± 0.6

TABLE III
Recovery of α-Tocopherol and Stigmasterol

Sample	α-Tocopherol		Stigmasterol	
	Added mg	Found mg	Added mg	Found mg
1	4.1	3.8	5.7	6.0
2	8.7	8.7	10.2	11.2

The most important variable in this determination is the chromatographic column. A satisfactory column must be prepared or the procedure will not yield proper results. We have found that not all batches of Gas Chrom Q will produce a satisfactory column. If a poor column is obtained from a batch of support, it is possible sometimes to rejuvenate this support by heating for 15 hr at 350 C. If after this treatment a poor column is still obtained, there is no recourse but to try a different batch of Gas Chrom Q.

All satisfactory columns gave a theoretical plate number of at least 4000 for the stigmasterol peak. This calculation might be used as one of the criteria for determining whether a column is functioning properly. The theoretical plate number of a chromatographic column was calculated using the equation $N = 16 (\frac{X}{Y})^2$, where: N = theoretical plate number; X = peak retention time, measured to the apex of the peak; and Y = peak width measured between the baseline intercepts of lines drawn tangent to the peak sides.

There is a wide variation in useful life of the chromatographic columns, some lasting only two weeks, others two months. This wide variation in column life cannot be attributed to any one factor.

ACKNOWLEDGMENT

Dmytro Bas assisted in these analyses.

REFERENCES

1. Analyst *84*, 361–362 (1959).
2. Beerthuis, R. K., and J. H. Recourt, Nature *186*, 373–374 (1960).
3. Carroll, K. L., and D. C. Herting, J. Assoc. Offic. Agr. Chemists *41*, 473–474 (1964).
4. Eisner, J., J. L. Iverson, A. K. Mozingo and D. Fristine, Ibid. *48*, 417–433 (1965).
5. Emmerie and Engel, Rec. Trav. Chim. *57*, 1351–5 (1938).
6. Libby, D. A., and A. J. Sheppard, J. Assoc. Offic. Agr. Chemists *47*, 371–376 (1964).
7. Peaau and Hardy, J. Pharm. Chem. *9*, 149 (1929).
8. Rozanski, A., Anal. Chem. *38*, 36–40 (1966).
9. Ishikawa, Seiji, Goichiro Katsui, Bitamin *31*, 445–448 (1965).
10. Slover, H. T., L. M. Shelley, T. L. Burks, J. Assoc. Offic. Agr. Chemists *44*, 161–166 (1967).
11. Wilson, P. W., E. Kodicek and V. H. Booth, Biochem. J. *84*, 524–531 (1962).

[Received July 1, 1968]