a fructosan-accumulating species (Smith and Groteluesch en, 1966), was used to evaluate this method for characterizing and quantitizing carbohydrate fractions in forage plants. Data indicated that roots of this species have a relatively high free fructose content. The increasing fructose/glucose (Cf/Cg) ratio corresponding with decreasing ethanol extractions beginning with the 92.5% hydrolyzed extraction indicated the presence of fructosans of varying chain lengths and the greatest quantity of fructose was extracted with 60% ethanol. These data corroborate the findings of Smith and Groteluesch en (1966). Fructosans in grasses are reported to be similar to the inulin type found in *Compositae*, but are thought to occur in chain lengths shorter than 35 fructofuranose residues linked 2:6 and terminated by a sucrose residue (Bacon, 1960; Edleman, 1960). In this experiment, fructose/glucose ratios increased with decreasing ethanol concentration indicating the presence of fructosans with average chain lengths of 1.05 (sucrose) at 92.5% ethanol extraction to 20.6 (inulin) at 0% ethanol extraction, in tall fescue roots.

Colorimetric determination of fructose in the presence of glucose can be used advantageously for differentiating these two sugars in plant extracts where they are present in greatest quantities. This method, in combination with the ethanol dilution extractions and acid hydrolyses, allows for routine quantitative description of carbohydrates in plant material, with the exception of starch. Because of the simplicity of this method, it can be used for routine determination of sugar components in forage samples, in particular the fructosan accumulating species.

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Received for review May 24, 1971. Accepted October 20, 1971. Contribution from the University of Georgia, College of Agriculture Experiment Stations, Georgia Station, as Journal Series Paper No. 1032.

Identification and Estimation of Tocopherols and Tocotrienols in Vegetable Oils Using Gas Chromatography–Mass Spectrometry

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A one-step method to estimate and identify TMS derivatives of tocopherols and tocotrienols using gas chromatography and mass spectrometry is described. Thin-layer chromatography is used as a pretreatment of unsaponifiable material when the critical isomers β- and γ-tocopherols are present together. The contents of individual tocopherols and trienols in oats, wheat germ, barley, soybean, and coconut oils are recorded. Wheat germ oil has a total tocopherol content of 212 mg/100 g of oil; oats, barley, and coconut oils have less than 3 mg/100 g of oil. Oats and barley oils are found to contain major amounts of α-tocotrienol, whereas coconut oil contains α-tocotrienol. The bulk of the tocopherols are distributed between α- and β-tocopherols in wheat germ oil and γ- and δ-tocopherols in soybean oil. Barley oil seems to possess almost all the known tocopherols and trienols. However, the presence or absence of δ-tocotrienol has not been determined due to nonavailability of the standard compound.

Separation of individual tocopherols from the nonsaponifiable material of oils and fats has been one of the major activities in this field. Of the eight tocopherols and trienols (α-, β-, γ-, and δ-, and their unsaturated counterparts, Figure 1), the positional isomers β- and γ-tocopherols are the most difficult to separate. Earlier, Quaife (1948) adopted a method to differentiate tocopherols under two main classes: α-tocopherol and non-α-tocopherols. This classification was made on the basis that β-, γ-, and δ-tocopherols reacted with nitrous acid to give a yellow nitroso derivative which could be determined by the colorimetric procedure of Emmerie and Engel (1939). Since the three nitroso derivatives have different extinction coefficients, the only disadvantage of this method was that the most important α-tocopherol was measured by difference.

The second method most popular during this period was the Dianisidine coupling reaction (Weisler *et al.*, 1947). The γ- and δ-tocopherols couple with diazotized *o*-dianisidine in alkali solution. The dye can be extracted and determined colorimetrically. However, the method is valid only when β-tocopherol is absent, but when present it is measured with α-tocopherol. With the discoveries of tocotrienols, the method became more complicated since 5,8-dimethyl tocotrienol (ε- or β-T-3) and 7,8-dimethyl tocotrienol (η- or γ-T-3) both gave the nitroso reaction, in addition 7,8-dimethyl tocotrienol (γ-T-3) coupled with dianisidine; therefore, its presence complicates the validity of an α- and γ-tocopherol determination.

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Techniques such as potentiometry (Karrer and Keller, 1939) and polarography (Beaver and Kaunitz, 1944) have been used to separate the four major tocopherols; however, β- was always measured with γ-tocopherol when both were present. Russell Eggitt and Ward (1953) successfully used reversed phase paper chromatography to separate tocopherols but the material had first to be rigorously freed of glyceride, carotenoids, and sterols by long chromatographic runs with the inherent danger of tocopherol destruction. Green and Marcinkiewicz (1959) used zinc carbonate impregnated paper to separate tocopherols and tocotrienols without extensive pretreatment. The well standardized two-dimensional quantitative paper chromatographic method of the British Analytical Methods Committee (1959) has been widely employed.

Currently, gas and thin-layer chromatographic methods are most commonly used. Wilson *et al.* (1962), and Slover and coworkers (1967, 1968, 1969) have used S.E.-30, Apiezon L, and QF1 Columns to separate all eight tocopherols and trienols, but still the most difficult β and γ isomers were measured together. To obtain a good separation, very careful preparation of the column was necessary, and the studies indicated that efficiencies of at least 400 to 450 theoretical plates per foot were needed. The isomeric β- and γ-tocopherols have been separated by thin-layer chromatography (Stowe, 1963). This method gave recoveries of 97–98%, with a synthetic mixture of α-, β-, γ-, and δ-tocopherols (Govind Rao *et al.*, 1965). Two-dimensional thin-layer chromatography was attempted by Pennock *et al.* (1964) to separate completely seven out of the eight known tocopherols and trienols, but their solvent system could not separate β-tocotrienols from γ-tocopherol. None of the above methods indicate the structure of the compound under identification, which must be identified either by comparison of R_f values on thin-layer chromatography or by their retention times on gas–liquid chromatography using standards.

In the present paper a procedure is described whereby the trimethylsilyl ether (TMS) derivatives of the unsaponifiable materials of oils and fats are subjected to combined gas chromatography–mass spectrometry. This procedure allows the structural determination and quantification of tocopherols and tocotrienols in one step. However, if both β and γ isomers were present simultaneously, a preliminary separation by thin-layer chromatography was necessary to eliminate the danger of estimating β- and γ-tocopherols together.

MATERIALS AND METHODS

Authentic samples of α-, β-, γ-, and δ-tocopherols were obtained from Eastman Kodak Co., Rochester, N. Y. α-Tocotrienol (α-T-3) was obtained from W. E. Scott, Hoffmann-La Roche, Inc., N.J., and β-tocotrienol (β-T-3) and ammonia stabilized latex (manufactured by Uni-Royal, Inc., "Lotol"), from which γ-tocotrienol (γ-T-3) was isolated, was kindly supplied by Hal T. Slover, Nutrition Research Division, U.S.D.A., Beltsville, Md. An authentic sample of δ-tocotrienol (δ-T-3) was not available for our present study. These samples were accurately weighed and dissolved in benzene so as to give 1 mg/ml concentration. An artificial mixture of these compounds was made and their purity was calculated after their separation on thin-layer chromatography (Govind Rao *et al.*, 1965).

Barley and soybean oils were solvent extracted in Soxhlet for 2 hr with shaking. Cold-pressed wheat germ oil was obtained from Viobin Laboratories, Monticello, Ill. Coconut oil was purchased from local market and oats (green grouts) oil was obtained from Quaker Oats Co., Chicago, Ill.

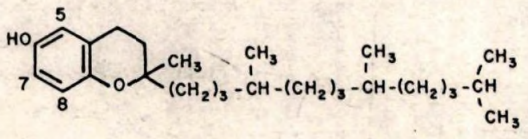
One gram each of the above oils, 4 ml of 5% pyrogallol solution in ethanol, and a few boiling chips were placed in a 150-ml round-bottomed flask fitted with a reflux condenser and heated on a water bath. When the mixture started boiling, the condenser was removed and 1 ml of aqueous potassium hydroxide (160 g dissolved in 100 ml of distilled water) was added slowly and refluxed for 5 min, cooled, and about 50 ml of distilled water was added. The nonsaponifiable material was extracted with peroxide-free diethyl ether. The ether extract was washed free of alkali, evaporated to dryness *in vacuo*, and the resulting unsaponifiables were weighed and diluted in benzene to give a concentration of 1 mg/ml.

Known amounts of these unsaponifiable materials were spotted on a 20-cm × 20-cm glass plate coated with supelcosil 12-B (Supelco, Inc., Bellefonte, Pa.) about 1 mm thick for preparative work and 250 μ thick for analytical work. About 1 ml of 0.1% alcoholic sodium fluorescein was added during preparation of plates to show up separated tocopherols and trienols as purple spots under ultraviolet light. Thin-layer chromatography was used to determine the purity of authentic compounds supplied to us and also to isolate the most difficult pair, β- and γ-tocopherols, when present together in the unsaponifiable materials of the representative vegetable oils under study.

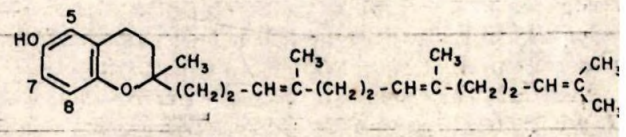
In the present study, the tlc technique was used as a preliminary pretreatment to ensure that β- and γ-tocopherol isomers are not present together and when present, as in the case of barley seed oil, two-dimensional tlc was carried out. Tocopherols and tocotrienols were separated on tlc, using chloroform in the first direction and a five component solvent system (petroleum ether 127, isopropyl ether 16, ethyl ether 1.5, acetone 6, and acetic acid 1.5) in the second direction. After developing the tlc plates, the purple tocopherol spots viewed under ultraviolet light were scraped, extracted with benzene, and converted into its corresponding TMS derivatives before being subjected to combined gas chromatography and mass spectral analysis.

Trimethylsilyl Derivatives. Trimethylsilyl ether (TMS) derivatives of both unsaponifiable material from vegetable oils and authentic samples of tocopherols and trienols after their separation on tlc were prepared by adding 0.1 ml o

TOCOPHEROL



TOCOTRIENOL



Position of Methyl Group		Tocopherol	Tocotrienol
5, 7, 8	Trimethyl	α	α_3
5, 8	Dimethyl	β	β_3
7, 8	Dimethyl	γ	γ_3
8	Monomethyl	δ	δ_3

Figure 1. Structures of tocopherol and tocotrienols

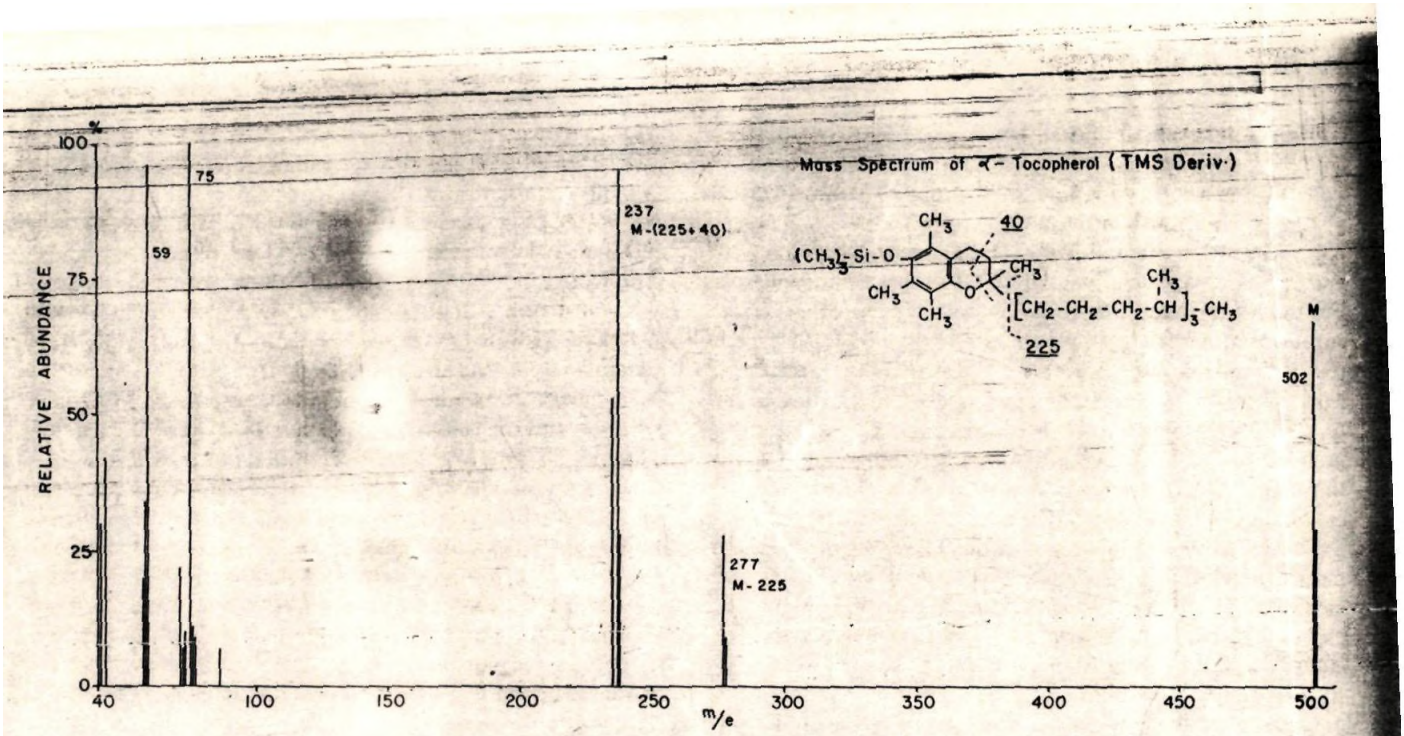


Figure 2. Mass spectrum of α-tocopherol–TMS derivative

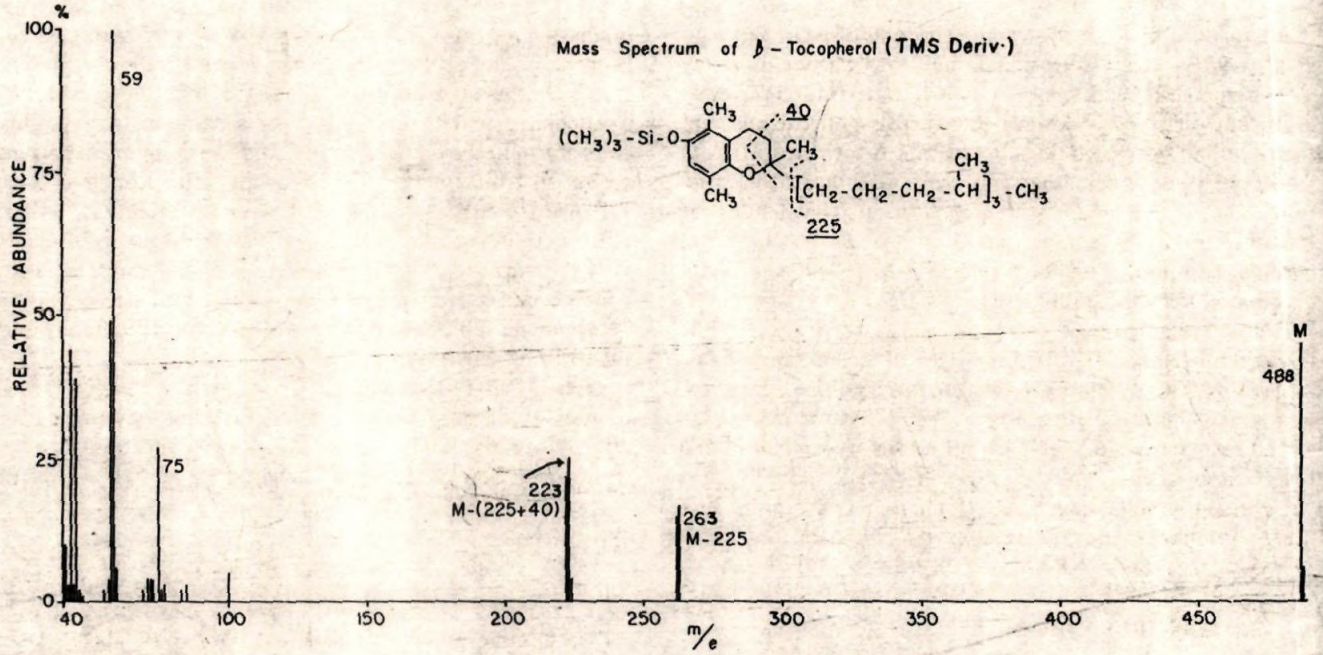


Figure 3. Mass spectrum of β-tocopherol–TMS derivative

Bistrimethyl Silyl Acetamide (BSA, Pierce Chemical Co., Rockford, Ill.) to sample containing about 50–75 μg and shaken well for 15 min before use. The TMS derivatives were prepared under nitrogen atmosphere and kept air tight under refrigeration until further use.

Combined Gas Chromatography and Mass Spectrometry. Approximately 3–5 μg of the TMS derivative was injected into a gas chromatograph (Aerograph Model 1200) with a hydrogen flame detector coupled to a RMU-6 Hitachi Perkin-Elmer double focusing mass spectrometer having a split ratio of 1:8. The chromatograph contained a 15-ft × 1/8-in. i.d. Stainless Steel Column packed with SE-30 on ultraphase (Pierce Chemical Co., Rockford, Ill.). The nitrogen carrier gas flow was maintained at 15 ml/min, the column temperature at 240°C, and the injection port at 260°C. Careful preparation of the column gave reproducible retention ratios, and the peak areas of each compound were also reproducible within ±0.8% of error for replicate injections using octacosane ($C_{28}H_{58}$) as internal standard. The separation of α-tocopherol from γ-tocotrienol was difficult, especially in the case of barley seed and coconut oils. We overcame this difficulty by slow temperature (150°C to 300°C at 6°C/min) programming of the gas chromatograph. While passing the gc effluent into the mass spectrometer, care was taken not to overload the column since the separations of α-tocopherol and β- and γ-tocotrienols were critical. The mass spectrum of each compound was computed to obtain the relative abundance of each peak. Authentic samples of tocopherols and tocotrienols were run to obtain standard spectra followed by the non-saponifiable materials of vegetable oils.

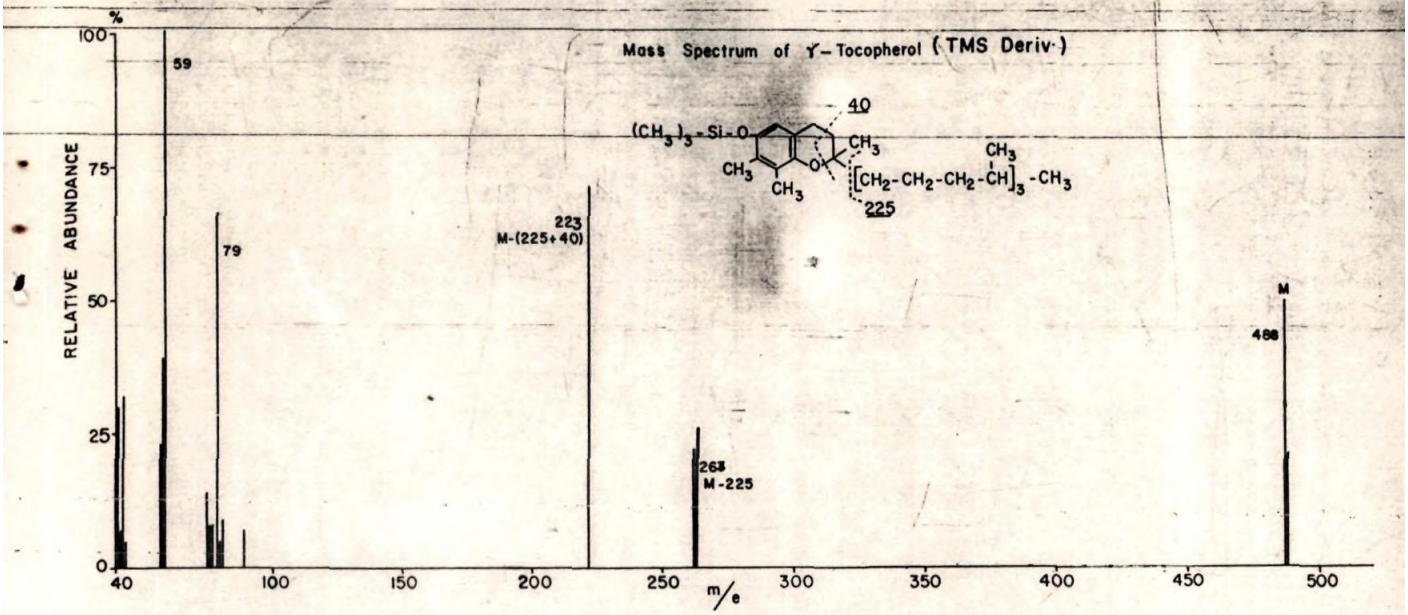


Figure 4. Mass spectrum of γ-tocopherol–TMS derivative

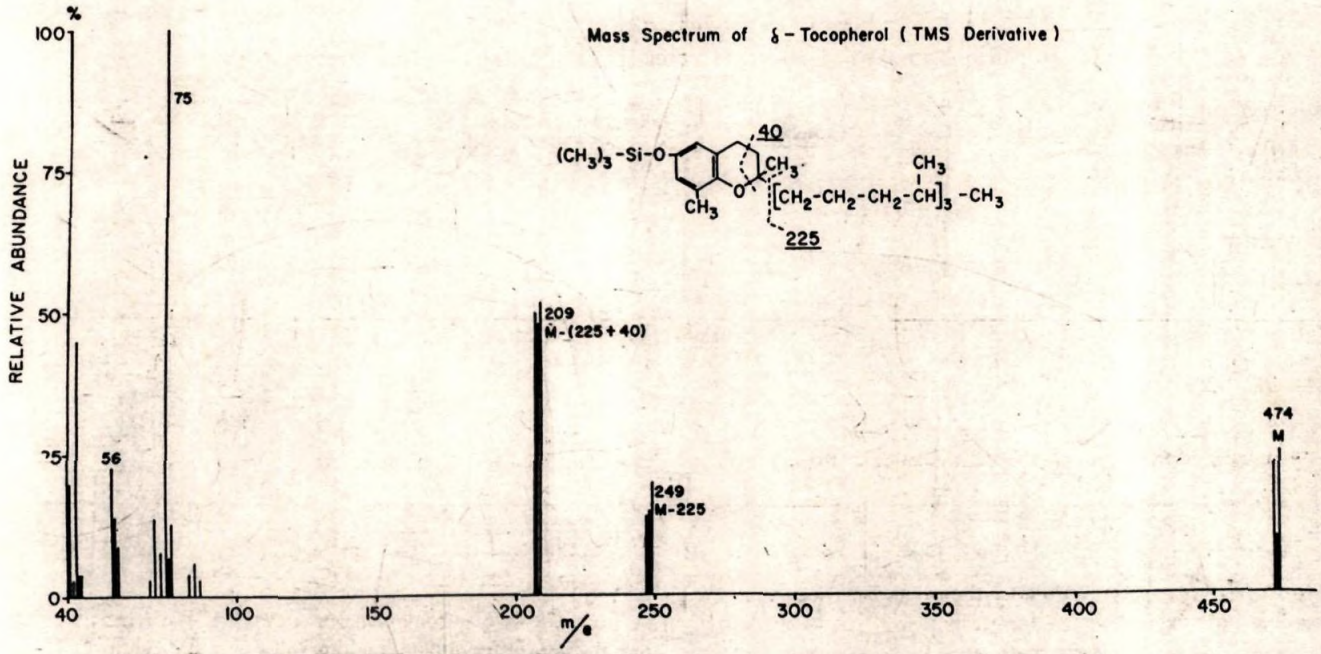


Figure 5. Mass spectrum of δ-tocopherol–TMS derivative

RESULTS

Table I shows the data obtained on quantitation of tocopherols and trienols of the oats, wheat germ, barley, soybean, and coconut oils. The total tocopherol content is expressed in mg/100 g of oil and the individual tocopherols and trienols as percent present. The choice of these oils is made so as to represent the presence of most of the tocopherols and trienols known. The presence or absence of δ-tocotrienol could not be determined, as the authentic standard was not available for our study. The quantitation of these compounds was done by gas chromatography, the areas of each peak were calculated by triangulation, and the amount of individual tocopherol was calculated according to the procedure described by Slover *et al.* (1968).

Oats, barley, and coconut oils contain small quantities of tocopherols. Oats contain mostly α-tocotrienol (58.3% of the total tocopherols present). Barley contains almost all the known tocopherols and trienols, of which 57.1% is α-tocotrienol. Coconut oil has mostly γ-tocotrienol (53.6%); wheat germ oil contains a high content of total tocopherols which seems to be distributed between α- and β-tocopherols. Soybean oil contains 61.9% γ-, 26.6 δ-, and 11.5% α-tocopherol; no trienols were found. The results reported are the average of three determinations which varied within ±1.0%.

The mass spectrum of the TMS derivative of α-tocopherol (Figure 2) is a simple spectrum which exhibits little fragmentation. The initial few peaks at *m/e* 75 are due to the dimethyl silanol [$(CH_3)_2$–SiOH] ion. The peak at *m/e* 277, *i.e.*, (M − 255), indicates the loss of a side chain ($C_{16}H_{33}$), and the peak at *m/e* 237, *i.e.*, (M − 255 + 40) originated from the cleavage

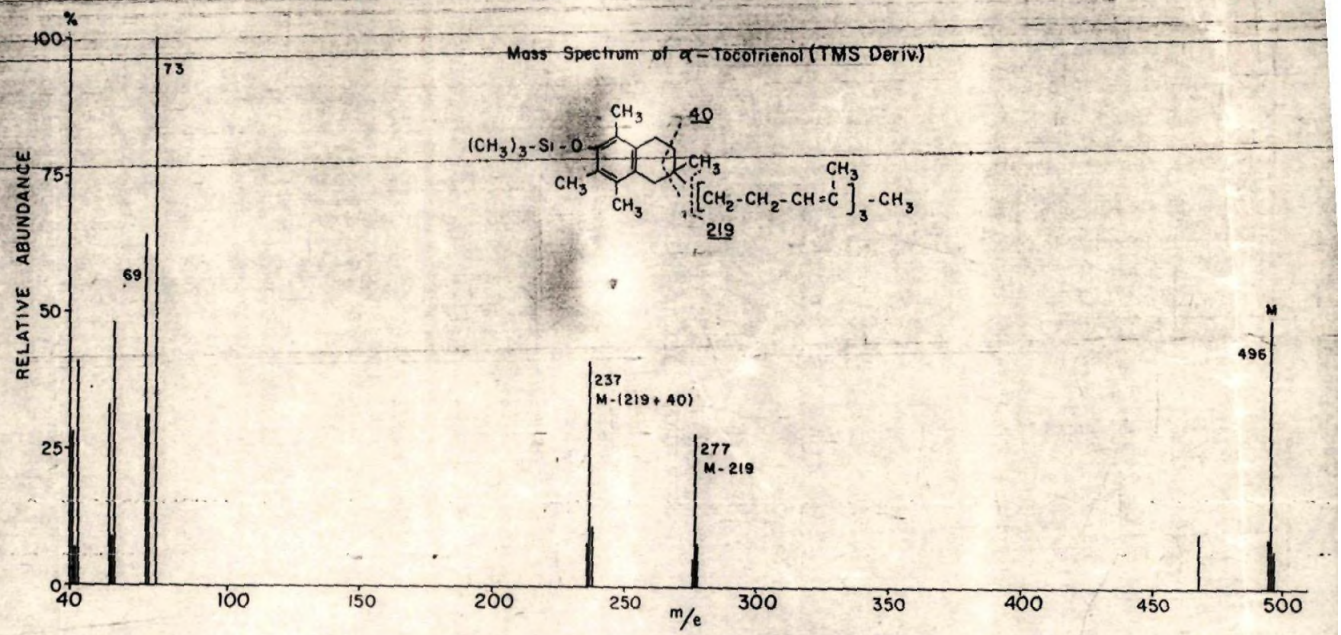


Figure 6. Mass spectrum of α-tocotrienol–TMS derivative

Table I. Individual Tocopherol Contents of Vegetable Oils (Average of Three Determinations)

Oil	Total tocopherol mg/100 g oil	Individual tocopherol, %						
		α-	β-	γ-	δ-	α_3-	β_3-	γ_3-
Oats	1.68	17.9[a]	6.0			58.3	17.3	
		(17.3–18.6)	(5.3–6.6)			(57.6–59.1)	(17.4–18.4)	
Wheat germ	212.00	51.9	38.1			4.0	6.0	
		(51.1–52.8)	(37.9–38.6)			(3.3–4.6)	(5.4–6.8)	
Barley	2.10	14.3	2.4	2.4	1.9	57.1	14.2	7.6
		(12.9–15.3)	(1.9–3.0)	(1.5–3.3)	(1.9–2.7)	(55.4–58.5)	(13.7–14.6)	(6.9–8.5)
Soybean	89.70	11.5		61.9	26.6			
		(10.4–12.3)		(60.7–62.6)	(25.8–27.3)			
Coconut	2.80	14.3		16.1		10.7	5.3	53.6
		(13.2–15.1)		(15.8–16.4)		(9.9–11.4)	(4.8–5.7)	(53.0–54.2)

[a] Figures on top are mean of three determinations. Figures in parentheses are minimum and maximum values.

of the side chain accomplished by the breakdown of chroman structure with hydrogen rearrangement and loss of a methyl acetylene $CH_3{-}C{\equiv}CH$ fragment in a fashion similar to that reported by Nair and Luna (1968) for the trifluoracetyl derivative of α-tocopherol.

The mass spectra of β- and γ-tocopherol derivatives (Figures 3 and 4) have similar fragmentation patterns, since these are positional isomers having the same molecular weights. The mass spectra of these isomers resemble those of α-tocopherol fragmentations, except that they differ by a mass number of 14. Similarly, the δ-tocopherol derivative (Figure 5) has a mass 14 less than β- and γ-tocopherols and 28 less than α-tocopherol.

In Figure 6 the peak at *m/e* 496 represents a molecular ion of TMS derivative of α-tocotrienol, which represents its molecular weight. The fragmentation pattern is similar to that of α-tocopherol, with only the difference in molecular weight which has 6 protons less than α-tocopherol due to the presence of three double bonds. Similar differences are noted in other tocotrienol derivatives (Figures 7 and 8) as in their saturated counterparts.

DISCUSSION

Since the two parent structures (Figure 1) of tocopherols and trienols differ only in the long side chain ($C_{16}H_{33}$ or $C_{16}H_{27}$), either saturated or unsaturated, the mass spectra of these closely related compounds show similar patterns of fragmentation breakdown but differ only in their molecular weights. For this reason, the molecular ions are shifted by a mass number of 6 between α-tocopherol and α-tocotrienol derivatives and similar changes occur in the subsequent compounds. No difference was observed between the positional isomers by mass spectrometry; however, the β and γ isomers may be distinguished by their infrared spectra, since they have characteristic absorptions between 7.5 and 9.8 μ (Morris and Haenni, 1962). β-Tocopherol has singlets at 8.1 and 8.65 μ, with shoulders at 8.47 and 8.55 μ and also doublets at 9.2 and 9.4 μ, whereas γ-tocopherol has two doublets at 8.05 and 8.25 μ, and 9.07 μ and 9.25 μ. Similar differences are also noted between β- and γ-tocotrienols, but the nmr spectra taken in this laboratory of β- and γ-tocopherols and their corresponding trienols are identical. Due to these difficulties, it would be easier to run a preliminary tlc to know whether they are

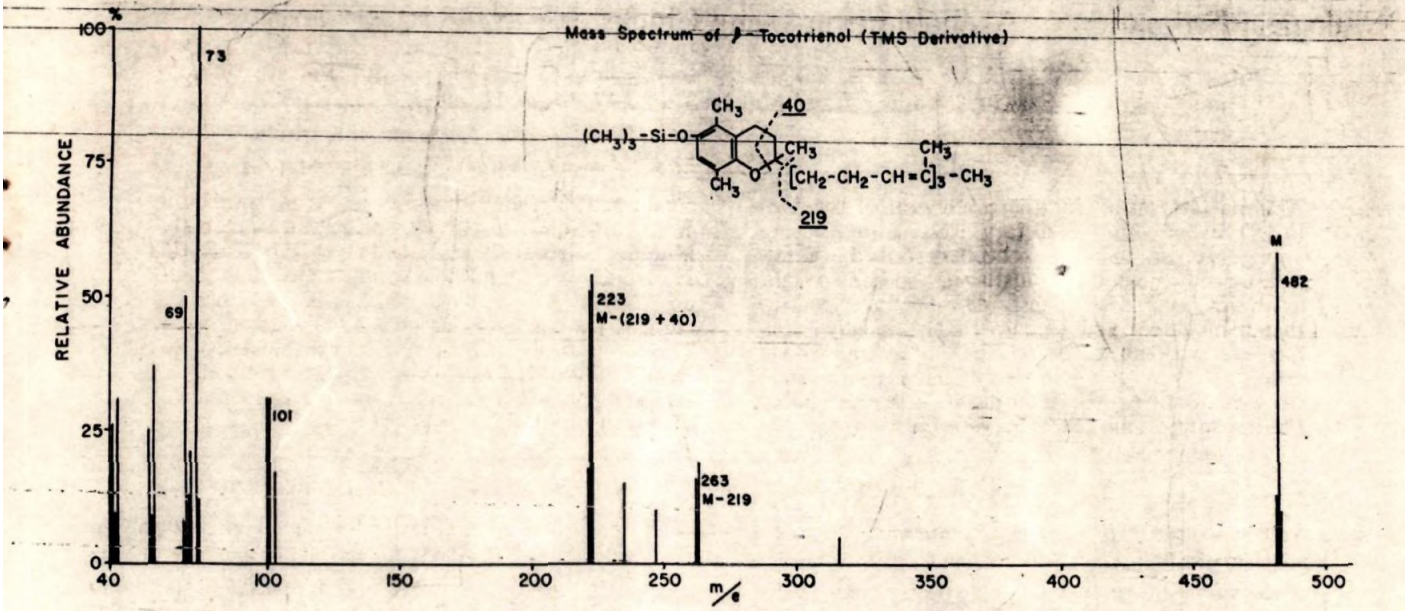


Figure 7. Mass spectrum of β-tocotrienol–TMS derivative

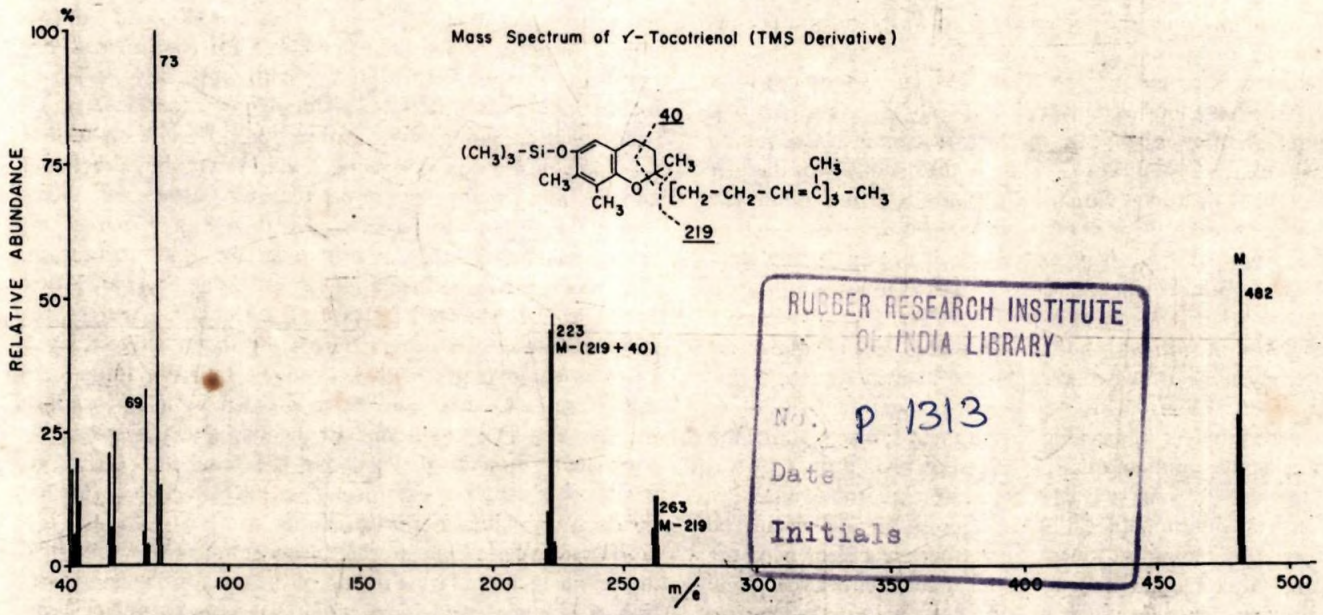


Figure 8. Mass spectrum of γ-tocotrienol–TMS derivative

present together; if so, it is advisable to isolate them and confirm the difference by infrared spectrophotometry.

ACKNOWLEDGMENT

The authors thank Everett Mayhood for mass spectra and Marilyn Sullivan for her assistance.

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Received for review May 21, 1971. Accepted September 9, 1971.